27th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology

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Dipartimento di Agraria, Università degli Studi di Napoli - Federico II

Proceedings of the 27th Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology

Università degli Studi di Napoli Federico II
Dipartimento di Agraria Portici (NA, Italy), 13th – 15th September 2023

This book collects the conference proceedings of the 27th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, held at the Department of Agricultural Sciences-University of Naples - Federico II from 13th to 15th September 2022. The goal of the conference is to gather PhD students from Italian universities whose projects deal with food-related topics to define the state of the art of Italian academic research in this area of study.

Keywords: Food Science, Food Technology, Microbiology, Biotechnology, Italian PhD Research, PhDFood 2023

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Prof Paola Piombino

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Scientific Committee

27th PhD workshop program

WEDNESDAY **SEPTEMBER 13th 2023**

11:00|13:00 Pre-event: **COSTAL** Meeting (Room 8)

13:00|14:30 **Registration & welcome coffee break (**Galoppatoio)

14:30|15:00 **Opening ceremony** (Room 2, in parallel streaming Room 7)

15:00|15:45 **Plenary lecture** (Room 2, in parallel streaming Room 7)
Chair: Silvana **Cavella**
Searching for the sustainability of food and drink industry **| Mauro MORESI**

15:50|17:10 **Parallel session 1: Food quality and processing** (Room 2)
Chairs: Cristina **Alamprese**, Bruno **Zanoni**

15:50|16:10 **Johnny Ciancetta** - University of Teramo
Formulation design approaches to increase stability, nutritional and healthy quality of frozen desserts

16:10|16:30 **Luigi Moriconi -** University of Camerino
Investigation at the boundaries between food processing and nutritional attributes of cereal-based products

16:30|16:50 **Marianna Tagliasco** - University of Udine
Innovative approach to design cereal-based product with low glycemic response

16:50|17:10 **Michela Pia Totaro** - University of Bari Aldo Moro
Technological approaches to improve the quality of meat products

15:50|17:10 **Parallel session 2: Microbiota, metabolome, health (**Room 7)
Chairs: Eugenio **Parente;** Marisa **Manzano**

15:50|16:10 **Qiuyu Lan** - University of Bologna Alma Mater Studiorum
Metabolomics to investigate the effects of treatments on food and of food consumption on health

16:10|16:30 **Giacomo Mantegazza** - University of Milano
Ready-to-eat food as a vehicle of microorganisms in the context of the *microbial deprivation hypothesis*

16:30|16:50 **Nadia Serale** - University of Bari Aldo Moro
Microbiota and metabolome in chronic non-communicable diseases

16:50|17:10 **Dimitra Tsoureki** - University of Torino
Dual approaches to investigate the infant food microbiome and *Listeria monocytogenes* behaviour under severe acidic conditions

17:10|17:50 **Coffee break** (Galoppatoio)

Poster viewing (Galoppatoio)

7th What For Award, Federalimentare

Video presentation of the selected final proposals (Room 2 and 7)

17:50|18:50 **Parallel session 3: Probiotics and Postbiotics (**Room 2)
Chairs: Teresa **Zotta**; Fabio **Minervini**

17:50|18:10 **Giulia Bisson -** University of Udine
Microbial exopolysaccharides as postbiotics for the development of new functional foods

18:10|18:30 **Maria De Sena** - University of Naples Federico II
Probiotic bacilli incorporation in foods: is really so easy?

18:30|18:50 **Irene Giordano** - University of Napoli Federico II
Metabolic attenuation of probiotics: a strategy for functional beverages development

17:50|18:50 **Parallel session 4: Authenticity and perception** (Room 7)
Chairs: Tullia **Gallina Toschi**; Emanuele **Boselli**

17:50|18:10 **Luigi Esposito** - University of Teramo
Dietary habits and biogenic amine exposure in Mediterranean populations: Insights from the italian aperitivo/happy hour context

18:10|18:30 **Giovanni Fiorile** - University of Napoli Federico II
Sensory methods ensuring authenticity and fostering Mediterranean fish

18:30|18:50 **Noemi Sofia Rabitti** - University of Milano
Individual variation in food perception and Implication in consumer preference of sustainable products

THURSDAY **SEPTEMBER 14th 2023**

09:00|09:45 **Plenary lecture** (Room 2, in parallel streaming Room 7)
Chair: Rossella **Di Monaco**
Chew on it: How oral breakdown of foods drives texture and flavour perception| **Markus STIEGER**

09:50|11:10 **Parallel session 5: Functional foods (**Room 2)
Chairs: Maria Cristina **Messia**, Monica **Anese**

09:50|10:10 **Irene Maria Grazia Custureri -** University of Reggio Calabria Mediterranea
Enrichment of extra virgin olive oil for the development of functional oil for special consumers

10:10|10:30 **Lorenzo Estivi -** University of Milano
Alternative strategies for the development of high-nutritional-value products from cereals and pulses

10:30|10:50 **Arianna Ressa** - University of Bari Aldo Moro
Novel insights on the functional and nutritional features of the foods based on cereals and legumes

10:50|11:10 **Stefano Tonini -** Free University of Bozen-Bolzano

Advancement and prospects of study of bioactive peptides during food fermentation

09:50|11:10 **Parallel session 6: Food systems sustainability** (Room 7)
Chairs: Matteo Mario **Scampicchio**, Lucilla **Iacumin**

09:50|10:10 **Chiara Demarinis -** University of Bari Aldo Moro
Development of biotechnological protocols for the valorization of alternative plant matrices as a strategy for the sustainability of agri-food systems

10:10|10:30 **Elisa Ghitti** - University of Milano
The role of root exudates in promoting beneficial interactions and rhizoremediation potential of polychlorinated biphenyls (PCBs)-degrading bacteria

10:30|10:50 **Ilaria Grigoletto** - University of Bologna Alma Mater Studiorum
Sustainable solutions in technology and quality control of olive oil

10:50|11:10 **Emanuela Lo Faro** - University of Modena and Reggio Emilia
Study and evaluation of strategies for replacing plastic materials with greener and eco-sustainable alternatives

11:10|11:40 **Coffee break** (Galoppatoio)

Poster viewing (Galoppatoio)

7th What For Award, Federalimentare
Video presentation of the selected final proposals (Room 2 and 7)

11:40|13:00 **Parallel session 7: Food safety** (Room 2)
Chairs: Rosalba **Lanciotti,** Teresa **Cirillo**

11:40|12:00 **Cristina Di Fiore** - University of Molise
Food products and microplastics: A call for qualification and quantification

12:00|12:20 **Federica D'Onofrio** - University of Teramo
Identification of virulence biomarkers in a *Listeria monocytogenes* ST7 strain through immunoproteomic and transcriptomic analysis

12:20|12:40 **Giulia Elli -** Free University of Bozen-Bolzano
Development of an electrochemical sensor to detect micro and nanoplastics in environmental and agri-food samples

12:40|13:00 **Valeria Tava** - University of Milano
Fusarium musae, a potential new food safety threat. Can a diseased banana be the source of a fungal disease for humans?

11:40|13:00 **Parallel session 8: Food by-product valorization** (Room 7)
Chairs: Spigno **Giorgia,** Martino **Forino**

11:40|12:00 **Beatrice Cellini** - University of Bologna Alma Mater Studiorum
Biotechnological valorisation of residues and by-products from agro-food industries

12:00|12:20 **Giuditta de Gennaro** - University of Bari Aldo Moro

Vegetable agri-food by-products: a source of functional ingredients for the production of high added value foods

12:20|12:40 **Silvia Donzella** - University of Milano
Biocatalytic modification of monoterpenes using waste-derived yeast cells

12:40|13:00 **Antonio Gattuso** - University of Reggio Calabria Mediterranea
Application of functional molecules recovered from bergamot by-products: development and improvement of food systems

13:00|14:30 **Lunch** (Galoppatoio)

Poster viewing (Galoppatoio)

7th What For Award, Federalimentare

Video presentation of the selected final proposals (Room 2 and 7)

14:30|15:15 **Plenary lecture** (Room 2, in parallel streaming Room 7)
Chair: Raffaele **Coppola**
Functional foods and human gut microbiota| **Maria DE ANGELIS**

15:20|16:40 **Parallel session 9: Food Biotechnology** (Room 2)
Chairs: Diego **Mora**; Stefania **Iametti**

15:20|15:40 **Annie Cecilia Castillo Ochoa** - University of Udine
Chimeric recombinant protein of *Brucella melitensis* and immunological evaluation for its possible use for the diagnosis of milk

15:40|16:00 **Amira Salim** - University of Sassari
The antimicrobial and antibiofilm activities of pomegranate peel phenolic compounds against foodborne pathogenic bacteria

16:00|16:20 **Lucia Sportiello** - University of Basilicata
Optimization of the extraction techniques using natural hydrophobic deep eutectic solvents for the recovery of biomolecules from food and food industry by-products

16:20|16:40 **Laura Troiani -** University of Parma
Leveraging Lactic Acid Bacteria for new sustainable processes

15:20|16:40 **Parallel session 10: Wine and fermented beverages** (Room 7)
Chairs: Luca **Rolle**; Viviana **Corich**

15:20|15:40 **Annina Caputo** - University of Napoli Federico II
Soil spatial variability at vineyard scale and relationship between grape elemental profile and enological characteristics of Aglianico grapewine in Taurasi DOCG area

15:40|16:00 **Antonino Pirrone** - University of Palermo
Application of non-conventional yeasts to improve the quality of innovative tropical fruit beverages

16:00|16:20 **Angelo Topo** - University of Udine
Role of closure and oxygen dissolved at bottling on white wine evolution: a multiparametric approach

16:20|16:40 **Cristian Alexis Galaz Torres** - University of Bologna Alma Mater Studiorum
Wine stability: implications of yeast mannoprotein additions prior to the bottling of red wine

16:40|18:30 **Coffee break** (Galoppatoio 16:40|17:00)

Poster viewing (Galoppatoio)

7th What For Award, Federalimentare
Video presentation of the selected final proposals (Room 2 and 7)

17:30|19:00 **PhD Coordinators** Meeting (Room 9)

CUVE Meeting (Laboratorio linguistico)

20:00|11:00 **Gala Dinner** (Pallacorda)

11:30 **Transfer**

FRIDAY **SEPTEMBER 15th 2023**

09:00|09:45 **Plenary lecture** (Room 2, in parallel streaming Room 7)
Chair: Paolo **Masi**
From research to business| **Luigi NICOLAIS**

09:50|10:50 **Session 11: 7th What For award | Selected candidates (**Room 2, in parallel streaming Room 7)
Chairs: Antonella **Verzera**; Maria Cristina **Nicoli**

09:50|10:10 **Lucrezia Angeli** - Free University of Bozen-Bolzano
Valorisation of South Tyrolean food products through the study of their antioxidant behaviour

10:10|10:30 **Nidhi Dalal -** University of Napoli Federico II
Finding Nemo's family: Enhancing NIR-based authentication of Mediterranean anchovies: the influence of spectra Pre-processing and machine learning techniques

10:30|10:50 **Michela Quiquero** - University of Molise
Mitigation strategies to reduce food-processing contaminants formation in Neapolitan pizza

10:50|11:10 **Coffee break** (Galoppatoio)

11:10|12:10 **Session 12: 7th What For award | Selected candidates** (Room 2, in parallel streaming Room 7)
Chairs: Marco **Dalla Rosa;** Donatella **Albanese**

11:10|11:30 **Francesco Ciuffarin -** University of Udine
Structuring oil for healthy and sustainable diets: the case study of the dried template approach

11:30|11:50 **Rohini Vijay Dhenge** - University of Parma
Modifications of vegetables subjected to conventional, innovative and nonthermal technologies

11:50|12:10	**Muhammad Rehan Khan** - University of Napoli Federico II Development of active antioxidant packaging to preserve the nutritional quality of minimally processed produce
12:10|12:25	**Awarding ceremony | SISS Best Sensory PhD Contribution Award** (Room 2, in parallel streaming Room 7)
12:25|13:00	**Awarding ceremony | 7th What For Award** (Room 2, in parallel streaming Room 7)
13:00|13:30	**Closing ceremony** (Room 2, in parallel streaming Room 7)
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Workshop contributions

1st year - PhD Dissertation Projects

Experimental fertilizers from food waste: a sustainable way to improve vegetative and productive performances of tomato plants

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In order to make the agricultural activity more sustainable, reducing its environmental footprint, this Ph.D. research project aims to valorise agri-food residues as soil fertilizers, evaluating their effect on vegetative and productive performances of tomato (*Solanum lycopersicum* L.). To reach these objectives, different strategies will be adopted, such as the addition of biochar and other fertilizers, obtained from agri-food waste, in different agricultural systems and *in vitro* tissue culture media.

Fertilizzanti sperimentali da rifiuti alimentari: un modo sostenibile per migliorare le prestazioni vegeto-produttive delle piante di pomodoro

Al fine di rendere l'attività agricola più sostenibile ridurne l'impronta ambientale, questo progetto di ricerca Ph.D. mira a valorizzare i residui agroalimentari per creare fertilizzanti del suolo, valutandone l'effetto sulle prestazioni vegetative e produttive di pomodoro *(Solanum lycopersicum* L.). Per raggiungere questi obiettivi saranno adottate diverse strategie, come l'aggiunta di biochar e altri fertilizzanti, ottenuti da rifiuti agroalimentari, in diversi sistemi agricoli e substrati di crescita per la coltura *in vitro*.

1. State-of-the-Art

In recent years, the modern agricultural system is facing the challenge of achieving food security goals, because of the growth of the world's population and the increasing demand for sustainable processes. Therefore, there are currently many studies in which agricultural residues are evaluated as biofertilizers or biostimulants and soil amendments, to exploit them as a resource and to reduce waste from primary production (Nattassha *et al.*, 2020).
A plant biostimulant is any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance or crop quality traits; lately, they are generating increasing interest and are gradually starting to replace synthetic fertilizers (Jardin *et al.*, 2015). Biochar is a soil amendment, obtained from pyrolysis, under anaerobic conditions, of agricultural by-products and organic wastes, widely used to improve soil's water-holding capacity and nutrient availability as well as different crops yield (Guo *et al*. 2021). A promising product is also the wood distillate (WD), a by-product of pyrolysis process for energy production, rich in different molecules such as esters, alcohols, acids, sugars, and phenols (Berahim *et*. *al*, 2011) which makes it able to act as a biostimulant for crops to increase biomass and fruit production.
Tomato (*S. lycopersicum* L.) is one of the most important crops in the world and even more in the Mediterranean area; for this reason, it is worth to evaluate alternative substrate compositions, both to improve plant vegeto-productive performances and to reduce the use of resource non-renewable and whose exploitation has a high environmental impact, such as peat (Gonnella *et al*., 2021).
Effects of the application of biochar and biofertilizers have been evaluated in numerous greenhouse and field studies, while few are the research exploring its influence on *in vitro* grown plants (Di Lonardo *et al.*; 2013; Wiszniewska *et al.*; 2023). Evaluating the effect of biochar added to the culture medium could open new perspectives for tomato micropropagation, improving *in vitro* plant response, reducing the high costs of tissue culture techniques and valorizing agricultural wastes.
Finally, WD has been used as biostimulant and to counteract fungi and bacteria, even at very low concentrations (Fedeli *et al.*, 2022) in several agricultural systems, but never as ingredient of culture media; instead, its use could be a way to replace synthetic additives, given its hormone like properties and its wealth in secondary metabolites (Gayathri *et al*., 2015).

2. PhD Thesis Objectives and Milestone

The main objectives of this PhD thesis project are listed below and outlined in the Gantt diagram given in Table 1:

A1) **Evaluation of the response of tomato plants at different types and concentrations of biochar added in the substrate, in soilless agriculture:** Morphological and physiological characterization of plants and fruits (A1.1), Physico-chemical analysis of plants and fruits (A1.2), Physico-chemical analysis of the substrate (A1.3).

A2) **Evaluation of the response of tomato plants at different concentrations of wood distillate added in the substrate, in soilless agriculture:** Morphological and physiological characterization of plants and fruits (A2.1), Physico-chemical analysis of plants and fruits (A2.2), Physico-chemical analysis of the substrate (A2.3).

A3) **Evaluation of the response of tomato plants at different types and concentrations of biostimulants added in the substrate, in soilless agriculture:** Morphological and physiological characterization of plants and fruits (A3.1), Physico-chemical analysis of plants and fruits (A3.2), Physico-chemical analysis of the substrate (A3.3).

A4) **Evaluation of biochar and biostimulants effect in tomato tissue culture:** Morphological characterization of *in vitro* grown plants (A4.1).

A5) **Bibliographic research, Paper and Thesis Preparation:** oral and/or poster communications, scientific papers and PhD thesis.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Evaluation of the response of tomato plants at different types and concentrations of biochar added in the substrate, in soilless agriculture***																								
A2)	***Evaluation of the response of tomato plants at different concentrations of wood distillate added in the substrate, in soilless agriculture***																								
A3)	***Evaluation of the response of tomato plants at different types and concentrations of biostimulants added in the substrate, in soilless agriculture***																								
A4)	***Evaluation of biochar and biostimulants effect in tomato tissue culture***																								
A5)	***Thesis and Paper Preparation***																								

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Validation of Sourdough Key Players using De Novo Synthetic Microbial Communities

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This PhD thesis research project is aimed at validating the key players of sourdough using new synthetic microbial communities (SMCs). Ultimately, this project envisions the application of robust sourdough SMCs in leavened baked goods instead of starter cultures, to optimize their fermentation, resulting in essential metabolites but maintaining their functionality by overcoming the perturbations of the fermentation process.

Convalida degli attori principali della pasta madre utilizzando le comunità microbiche sintetiche de novo

Questo progetto di tesi di dottorato ha lo scopo di convalidare i principali attori della pasta madre utilizzando nuove comunità microbiche sintetiche. In definitiva, il progetto prevede l'applicazione di tali comunità nei prodotti lievitati da forno al posto delle tradizionali colture starter, per ottimizzare la fermentazione e produrre metaboliti essenziali ma al contempo conservare la loro funzionalità nonostante le perturbazioni insite alla fermentazione.

1. State-of-the-Art

Microbiomes in food environments are complex, just like in the human gut. Today, steering food fermentations with starters (monocultures) alone is ecologically unsound and minimalistic. Sourdough fermentation exploits a complex microbial community (Arora *et al*., 2021). Recent studies carried out by Calabrese *et al*. (2022) revealed that a fermentome-driven sourdough fermentation (a consortium of about 8 strains) is more sustainable compared to the traditional starters. The consortium includes dominant, sub-dominant and satellite microbial players with complementary and unique metabolic functions, which ensure the stability and resilience of a mature sourdough. Sourdough microbial communities are typically composed of lactic acid bacteria such as *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum*, *Pediococcus pentosaceus* and *Furfurilactobacillus rossiae*, as well as yeast species such as *Saccharomyces cerevisiae* (Gobbetti *et al*., 2014). Metaomics analyses have shown that these communities may produce various metabolites that may have beneficial effects on the human gut microbiome, including short-chain fatty acids and antimicrobial peptides (Da Ros *et al*., 2021).
Mechanistic fermentation studies are complicated since observed functionalities result from diverse metacommunities rather than single species (Friedman *et al*., 2017). Coupled with metaomics, one of the complexity-reducing strategies developed to explain not only the compositional characteristics, but also the mechanistic causation influencing the emergent structural and functional traits of microbial communities is the use of Synthetic Microbial Communities (SMCs) (Calabrese *et al*., 2022; Karkaria *et al*., 2021). The SMCs approach is an emerging technique that involves co-culturing multiple taxa under well-defined conditions to mimic the structure and function of a microbiome. The metaomics approach has emerged as a powerful tool for studying complex and dynamic microbial consortia. It involves the simultaneous analysis of multiple types of biomolecules (DNA, RNA, proteins, and metabolites) to gain a comprehensive understanding of the functional and structural properties of microbial communities.
Overall, the current state-of-the-art reveal the need for further research to fully understand the mechanistic processes of sourdough microbiome. The recent study (Calabrese *et al*., 2022) did not reveal whether the robustness of sourdough metacommunities is strain- or species-specific. Therefore, this research seeks to validate the key players of sourdough using new SMCs composed of substitute strains and species. Overall, this study will change the paradigm and introduce theoretical foundations for guiding food fermentations.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Literature review and preliminary work**: the first months of the PhD have been dedicated to review of the state-of-the-art relevant to this thesis (A1.1). Pure isolates of the bacteria and yeast strains needed for the SMC construction will be obtained and confirmed after DNA extraction, standard PCR and Sanger's

sequencing (A1.2).

A2) **Construction of new sourdough synthetic microbial communities**: Wheat flour hydrolysate (WFH) will act as the model medium (A2.1). Autochthonous strains isolated from sourdoughs all around the world will be used. A total of eighteen (18) SMCs will be constructed in WFH (A2.2). This will comprise of new species/strains substituting the dominant, sub-dominant and satellite species/strains as defined by the prior research of Calabrese *et al*. (2022).

A3) **Competition experiments between dominant and sub-dominant lactic acid bacteria**: members from the most performing SMCs from M2 will be evaluated in pairs (one dominant with one sub-dominant) for specific interspecies competition assays. One dominant and one sub-dominant will be inoculated at 107 cfu/ml each in WFH, growth kinetics will establish the end of exponential phase. One per cent of the co-culture at the exponential phase of growth will be inoculated again in WFH and growth kinetics will be monitored for the total of 5 days (Janßen *et al*., 2018) (A3.1). Quantitative PCR (qPCR) will be used to determine the absolute abundances of the species during the interactions, metatranscriptomics and metabolomic analyses will be used to evaluate the metabolic interaction of the selected dominant and sub-dominant strains. Total number of all possible interactions for the members of the most performing new SMCs will be 6 and time points will be 3 (0, 2 and 5 days). Total number of samples will be 18 and analysed in duplicates (A3.2).

A4) ***In situ* experiment for stability and robustness of new SMCs**: to complete the design and validate the approach explained in A1 and A2, the most performing SMCs will be also evaluated under *in situ* conditions. Strains will be inoculated according to their abundance classification (core dominant, core sub-dominant, etc.) in specific cell densities in water and flour and will be fermented according to the conditions established for SDGlobal (A4.1). After maturation, the new SMC sourdough will be daily propagated for 30 days and each 10 days samples will be evaluated for the persistence of strains and their cell densities using qPCR. The same approach will be used for the most performing SMC that included *Kazachstania humilis* without *S. cerevisiae*. The goal is to evaluate if S. cerevisiae will find its way to appear during the daily propagations and consequently dominate over *K. humilis* (A4.2).

A5) **Research publications and thesis**: writing and editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Preliminary Work***																								
	1) State-of-the-Art Review																								
	2) Pure Culture Isolation																								
A2)	***New Sourdough SMCs Construction***																								
	1) Preparation of WFH Media																								
	2) SMCs Construction in WFH																								
A3)	***Competition Experiments***																								
	1) SMCs Interspecies Competition																								
	2) qPCR and Meta-Omics Analyses																								
A4)	***In Situ Experiments***																								
	1) SMCs *In Situ* Assay																								
	2) Stability and Robustness Analyses																								
A5)	***Research Publication and Thesis***																								

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Study of Chemical and Sensory Markers for Precision Oenology as a Component of the Decision Support System (DSS) for Wineries

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This PhD thesis research project aims to study chemical and sensory markers that wineries can use in a DSS to improve wine production and quality. Precision oenology, which optimizes winemaking operations using data-driven methodologies and DSS, is one of the two sides of aspects connecting the right product for the right consumer. The other side would be the development of non-invasive method for fingerprinting bottled wine products to ensure their authenticity and consumers expectations. The project will be developed in collaboration with wineries and providers of analytical non-invasive devices.

Studio di marcatori chimici e sensoriali per l'enologia di precisione come componente del sistema di supporto decisionale (DSS) per le aziende

Questo progetto di ricerca per la tesi di dottorato mira a studiare i marcatori chimici e sensoriali che le aziende vinicole possono utilizzare in un DSS per migliorare la produzione e la qualità del vino. L'enologia di precisione, che ottimizza le operazioni di vinificazione utilizzando metodologie guidate dai dati e DSS, è una delle due facce degli aspetti che collegano il prodotto giusto al consumatore giusto. L'altro aspetto è lo sviluppo di un metodo non invasivo per la rilevazione dell'impronta digitale dei prodotti vinicoli imbottigliati, per garantirne l'autenticità e le aspettative dei consumatori. Il progetto sarà sviluppato in collaborazione con aziende vinicole e fornitori di dispositivi analitici non invasivi.

1. State-of-the-Art

Wine production is a multi-stage process that starts with grape growth and harvest, and continues through fermentation, ageing, and bottling. Numerous elements, such as grape variety, *terroir*, viticulture practices, winemaking methods, and ageing circumstances, have an impact on the wine's quality and sensory qualities (Reynolds, 2022). Precision oenology is a burgeoning field of study that employs chemical and sensory markers to analyze and monitor different facets of wine production. Winemakers can make informed decisions regarding grape selection, fermentation techniques, maturation, and overall wine quality by identifying specific markers (Poggesi et al., 2021). As the wine industry strives to produce high-quality wines, the application of precision oenology has become increasingly vital. However, consumers and manufacturers alike continue to worry about the authenticity of wine sold in bottles. Financial costs for producers and damage to consumer trust can occur from fraudulent tactics like adulteration, mislabeling, and counterfeiting (Popović et al., 2021, Merkytė et al., 2020b). Therefore, it is important to create technologies for fingerprinting bottled wine products to satisfy consumers' demands for authenticity. Numerous studies have focused on the development of analytical methodologies for wine analysis. Typically, these techniques involve the identification and quantification of chemical compounds in wine samples. Liquid chromatography coupled with mass spectrometry (LC/MS) and Gas chromatography-mass spectrometry (GC-MS) are the common techniques for identifying non-volatile and volatile organic compounds in wines, which can reveal large information on the winemaking technology, grape variety, and vintage (Lukić et al., 2022, Merkytė et al., 2020a). Recently, non-invasive technologies, such as near-infrared spectroscopy (NIRS) and mid-infrared spectroscopy (MIRS), have acquired popularity due to their ability to enable rapid and non-destructive analysis of wine samples. These techniques can be used to learn key information about the wine, using chemical and sensory markers, such as its vintage, grape variety, and place of origin. Non-invasive procedures significantly reduce wine sample damage, allowing for repeated evaluations without harming product quality. In addition, changes in the molecular vibrations of chemical compounds in wine samples can allow the identification and quantification of diverse substances (Hu et al., 2019). Moreover, these methodologies are being already used to reveal the chemical composition of wine, such as its sugar and acid content, as well as the presence of specific compounds, such as tannins and flavonoids. Non-invasive chemical and sensory marker studies will assist create a comprehensive wine fingerprint database to match the right product for the right consumer. The DSS can use the database to optimize winemaking operations and improve wine quality. This database may additionally include wine's provenance, vintage, grape variety, chemical, and sensory markers. which can help to provide accurate product information and help consumers buy the right wine product. In addition, an exhaustive database can serve as a reference for future analyses of wine, which can aid in the detection of wine fraud.

2. PhD Thesis Objectives and Milestones

The main objective of this research proposal is to investigate the chemical and sensory markers and use of non-invasive techniques to optimize winemaking practices and improve wine quality and integrate those techniques into DSS.

A1) **Chemical Analysis of collected samples from wineries:** Wine samples from several wineries will be gathered to identify and quantify aroma compounds and their precursors in bottled wine samples using advanced analytical techniques, such as GC-MS, GCxGC-MS, and LC-MS. The effectiveness of different non-invasive fingerprinting techniques for wine products will be investigated. These methods will include spectroscopic techniques; specifically, near-infrared spectroscopy using the portable spectrometer.

A2) **Identification of Potential Markers:** To identify potential markers or combinations of markers that can be used to validate the authenticity of wine products. The markers discovered will be determined according to their sensitivity, specificity, and robustness.

A3) **Method Development and Validation:** To develop and validate a reliable method for applying the identified markers by using multivariate statistical approaches. The approach will be tested on a variety of wine products from various areas and vintages. To assess its efficacy, the created method will be compared to existing methods for wine authenticity from a supply chain traceability perspective.

A4) **Addition to Decision Support System (DSS):** To advise the design of a DSS to help wineries streamline their processes and produce higher-quality wine. Winemaking procedures like grape selection, fermentation, ageing, and blending tactics will be informed by data because of the DSS's integration of chemical and sensory markers revealed in the research.

A5) **Writing and Editing** the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Chemical Analysis***																								
	1) Advanced Analytical Techniques																								
	2) Non-invasive Techniques																								
A2)	***Identification of Potential Markers***																								
	1) Potential Markers Identification																								
	2) Sensitivity and Specificity																								
A3)	***Method Development***																								
	1) Method Validation																								
	2) Efficacy Assessment																								
A4)	***Addition to DSS***																								
	1) Design of DSS																								
	2) Integration of Identified Markers																								
A5)	***Thesis and Paper Preparation***																								

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Development of Sensing Solutions for Evaluation of Deposited Pesticides in Fruits

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This PhD thesis research project is aimed to ensure environmental sustainability as well as the safety in fruits by developing sensing solutions for the evaluation of deposited pesticides in fruits. This is two-fold study, and the first step involves the development of better protocols and methods to evaluate pesticide deposition. Secondly, effective detection methods that provide easy, quick, economical, and environmental friendly on-site detection of pesticides even at trace levels will also be developed. Furthermore, both methods will be validated, and their application will be carried out in the lab as well as in the field.

Sviluppo di soluzioni di rilevamento per la valutazione dei pesticidi depositati nella frutta

Questo progetto di ricerca di tesi di dottorato ha lo scopo di garantire la sostenibilità ambientale e la sicurezza nei frutti sviluppando soluzioni di rilevamento per la valutazione dei pesticidi depositati nei frutti. Questo è uno studio duplice e il primo passo prevede lo sviluppo di protocolli e metodi migliori per valutare la deposizione di pesticidi. In secondo luogo, saranno sviluppati anche metodi di rilevamento efficaci che forniscano un rilevamento in loco facile, rapido, economico e rispettoso dell'ambiente dei pesticidi anche a livello di tracce. Inoltre, entrambi i metodi saranno validati e la loro applicazione sarà effettuata sia in laboratorio che sul campo.

1. State-of-the-Art

Fruits and vegetables are a substantial part of a healthy balanced diet providing vitamins, minerals, polyphenols, and dietary fibre to the consumers leading to a healthy lifestyle (He et al., 2023). The increased health awareness among consumers, rapid urbanization, and changed lifestyle patterns has dramatically skyrocketed the demand for fruits and vegetables globally. In order to meet the demand, farmers apply pesticides excessively to increase the yield (He et al., 2023; Philippe et al., 2021). Many systematic studies have also indicated that almost half of the pesticides applied to crops enter the environment by contaminated soil, water, and air from the site of application or field (Schleiffer and Speiser, 2022). Thus, Regulatory authorities are strictly monitoring pesticide deposition to evaluate the performance of agricultural sprayers and bring required changes to reduce the adverse impacts of spray drift on the environment and to lower pesticide waste (Munjanja et al., 2020). Pesticide deposition can be investigated by using various tracers solutions such as fluorescent tracers, colorimetric tracers, and metal ion tracers (Srinivasarao et al., 2021). Fluorescent tracers are extensively used because they are economical, less harmful, practical and highly sensitive in comparison to other tracers (Zhang et al., 2020). The deposition assessment by sensor provides benefits over direct sampling by reducing time and labor costs and providing information on the spray drift (Qin and Chen, 2023). The development of sensing solutions for assessing spray drift has become a popular trend due to advancements in sensor technology (Li et al., 2022). Thus, there is a need to find sensing solutions for the evaluation of pesticides deposited on fruits to ensure environmental sustainability and safety in fruits. Right after the application of pesticides, their residues stay in the food, but the level is kept on decreasing due to their breakdown depending upon the type of food, type of pesticide used, application method, and post-harvest processing of food (Sindhu and Manickavasagan, 2023). Excessive exposure to chemical pesticides may cause diabetes mellitus, neurological disorders (Parkinson's disease & Alzheimer's disease), reproductive syndromes, respiratory issues, cancer, and oxidative stress (Umapathi et al., 2022). Keeping in view the associated health risk, Codex Alimentarius Commission has defined Maximum Residue Limits (MRLs) for all pesticides to protect human health and promote international fair trade (Sindhu and Manickavasagan, 2023). Thus, many attempts and investigations were carried out to reduce human exposure by restricting pesticide application and reducing the level of MRLs in food commodities. Conventionally, many methods such as atomic absorption spectrometry, High-Performance Liquid Chromatography, Liquid chromatography-mass spectrometry (LC-MS), Gas Chromatography, gas chromatography-mass spectrometry (GC-MS), Surface-enhanced Raman spectroscopy (SERS), capillary electrophoresis, and ELISA were employed for the detection of pesticides in various food commodities. However, high cost, greater time consumption, the requirement of expert personal, complicated pre-treatment of samples, and large instruments limit the application of these methods for on-site and quick detection despite being precise and highly sensitive methods (Schleiffer and Speiser, 2022). This highlights a dire need to

develop effectual analytical methods that could provide easy, quick, economic, and environmental friendly detection of pesticides even at trace levels.

2. PhD Thesis Objectives and Milestones

The project aims to assess the correlation between the amount of deposited pesticides and residues on fruits. So, the amount of pesticide deposited during spray will be evaluated and correlated to the residues on the fruit. Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Literature review**

A2) **Optimization and development of better protocols and methods to evaluate pesticide deposition:** The preliminary work starts with available instruments in the laboratory for the investigation of pesticide deposition. Moreover, the sensing methods to evaluate the pesticide deposition will be developed and tests will be conducted in the wind tunnel in the agroforestry and innovation lab by employing anti-drift nozzle and air-assisted spraying techniques.

A3) **Development of sensor-based solutions for the detection of pesticides in fruits:** The second year of the research will focus on the development of a sensing solution for pesticide detection in fruits. This work will be done in close collaboration with the Sensing technology lab. This second stage will be the next step toward the proposed study aim and will provide an alternative to a conventional method for pesticide detection in fruits.

A4) **Validation and application** of the sensing solutions developed during the first and second stages.

A5) **Writing and editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8		9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Literature review***																									
A2)	***Optimization and development of better protocols and methods to evaluate pesticide deposition***																									
A3)	***Development of sensor-based solutions for the detection of pesticides in fruits***																									
A4)	***Validation and Application of the sensing solutions***																									
A5)	***Thesis and Paper Preparation***																									

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Evaluation of Exposure to Microplastics and Nanoplastics Associated with the Consumption of Clam *Chamelea Gallina*

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This PhD thesis research project aims to develop analytical protocols for the determination of microplastics (MPs) and nanoplastics (NPs) in the clam *Chamelea Gallina*, through two instrumental techniques, the Raman spectroscopy and Pyrolysis coupled with gas chromatography-mass spectrometry (Py-GC/MS), that provide morphological and chemical information. The data obtained will allow to carry out a preliminary evaluation of the human exposure to the MPs/NPs taken through the diet, deriving from the consumption of this mollusc.

Valutazione sull'esposizione a microplastiche e nanoplastiche associata al consumo di vongola *Chamelea Gallina*

Questo progetto di tesi di dottorato mira a mettere a punto protocolli per la determinazione di microplastiche (MP) e nanoplastiche (NP) nella vongola *Chamelea Gallina*, mediante due tecniche strumentali, la spettroscopia Raman e la Pirolisi accoppiata a gas cromatografia-spettrometria di massa (Py-GC/MS) che forniscono informazioni di tipo morfologico e chimico. I dati ottenuti permetteranno di effettuare una valutazione preliminare dell'esposizione umana alle MP/NP assunte attraverso la dieta, derivante dal consumo di questo mollusco.

1. State-of-the-Art

Microplastics (MPs) are polymer particles with dimensions between 0.1 µm and 5 mm, ubiquitously dispersed in the environment and present in various forms including fibers and fragments. Their further fragmentation leads to the formation of nanoplastics (NPs) with dimensions up to 1 nm. The presence of these plastic particles has aroused considerable interest in recent decades due to their toxicity detected in marine organisms, and their potential role as a vehicle for other pollutants, thus increasing the exposure and facilitating the entry of substances toxic to humans, being at the top of the food chain.
The bivalve molluscs, given their nature of filter feeding, ingest many substances, including the MPs/NPs, which accumulate between tissues and internal organs based on the size. This feature allows them to be used as indicators of the healthiness of the marine environment and therefore possible to think of their use as bioindicators for plastics contamination (Ding J et al, 2021). Some protocols for the analysis of the MPs content in mussels that use different analytical approaches are available in literature (Pinto da Costa J et al, 2019).
These protocols involve the use of non-destructive techniques such as Raman spectroscopy, capable of characterizing the MPs by shape and size, as well as identifying them by comparing the spectrum obtained with characteristic reference spectra, optimizing a series of parameters such as the laser power, the number of scans and the exposure time.
Another technique that is recently taking hold for the analysis of MPs/NPs is pyrolysis coupled to gas chromatography-mass spectrometry (Py-GC/MS), a structural investigation technique that involves thermal degradation with temperatures above 500 °C and characterization of microplastics and any additives through pyrolysis products (Ishimura T et al, 2021).
Furthermore, the choice of analytical technique becomes fundamental in this case according to the information to be obtained; on the one hand, in fact, spectroscopic techniques make it possible to obtain information on the size and shape of the microplastics, while thermodegradation techniques allow to exploit lower sensitivities by analysing a minimum amount of sample, losing the morphological information.
Chamelea Gallina is a bivalve mollusc that feeds by filtering the surrounding waters and grows on the seabed. it is highly appreciated both in Italy and in the rest of Europe and the use of this food, consumed completely without removing internal organs, can constitute a source of contamination from microplastics. Although molluscs are among the foods most taken into consideration when it comes to contamination in the marine environment, there is still little data available on the number of MPs present, necessary to evaluate the risk of exposure associated with the consumption of the clam *Chamelea Gallina* (Gedik K, Gozler AM, 2022).
The proposed research project aims to fill the lack of analytical methods for the extraction, identification, and quantification of MPs/NPs in *Chamelea Gallina* clams. The use of different instrumental techniques will allow to obtain combined information on physical characteristics (color and size of the particles) and chemical characteristics (mass of the particles, or of the relative thermodegradation products). Finally, the application of

validated protocols on real samples will provide data on MPs/NPs contamination levels, filling the lack of data in the literature and allowing a first assessment of exposure to these contaminants with their intake.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Sampling planning* of the *Chamelea Gallina* clams in Abruzzo, Molise and Marche region** considering different parameters as collection areas and fishing block periods, and the consumption of the product (A1.1). The number of samples must be sufficient to be representative and must take place over 12 months (A1.2).

A2) **Analytical protocol** for the determination of the MPs/NPs with Raman spectroscopy** which will include the optimization of the microplastics extraction protocol from the matrix (A2.1), the instrumental method for the identification of polymers by varying key parameters (laser power, number scans and exposure time) (A2.2) and the analysis of the samples of clams (A2.3).

A3) **Analytical protocol for the determination of the MPs/NPs with Py-GC/MS** which will be conducted using the extraction method used for spectroscopy analysis, adapted for the preparation of the sample to pyrolysis (A3.1). The parameters to be optimized will concern the pyrolysis process and the identification of microplastics through the characteristic pyrolysis products and with certain values of *m/z*, as well as the acquisition parameters in mass spectrometry (A3.2). After the validation of the analytical protocol, the analysis of the samples of clams will be made (A3.3).

A4) **Definition of the levels of contamination of the *Chamelea Gallina* clams of MPs/NPs** by developing the data obtained by the two complementary analytical techniques (A4.1) and preliminary evaluation on the risk of exposure to MPs/NPS associated with the consumption of *Chamelea Gallina* clams (A4.2).

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

*The Sampling planning (A1.1) will end in June 2023, while the collection of samples will begin in July 2023 (first year of the PhD thesis project) and will finish in June 2024.

** The extraction protocol (A2.1) and instrumental optimization (A2.2) will be conducted during the first year of the PhD thesis project.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Sampling of Chamelea Gallina***																								
	1) Sampling planning																								
	2) Collection of samples																								
A2)	***Optimization of the Raman spectroscopy analytical protocol***																								
	1) Extraction protocol																								
	2) Instrumental optimization																								
	3) Samples analysis																								
A3)	***Optimization of the Py-GC/MS analytical protocol***																								
	1) Extraction protocol re-optimization																								
	2) Instrumental optimization																								
	3) Samples analysis																								
A4)	***Definition of contamination levels***																								
	1) Data elaboration																								
	2) Risk evaluation																								
A5)	***Thesis and Papers preparation***																								

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Yeast derivatives for precision oenology: emerging and sustainable application for wine production (WInnY)

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Yeast derivative products (YDPs) have been increasingly used in the wine industry thanks to the many needs they can meet. YDPs can be used as nutrient sources for the fermentation processes, as stabilizing agents and for improving the sensory characteristics of wines. This PhD project aims to find possible relationships between their chemical composition of the YDPs and their function. Several commercial YDPs will be characterized and they will be assayed as fermentation enhancers, stabilizing agents and as antioxidant during the wine aging. Thus, this proposal will provide important information to YDP producers who can improve their production methods and the precision of their products. In this way, the winemakers could benefit of more specific and effective YDPs.

Derivati di lievito per l'enologia di precisione: applicazione sostenibile ed emergente per la produzione enologica (WInnY)

I derivati di lievito (YDP) sono sempre più utilizzati nell'industria enologica grazie alle molteplici esigenze che possono soddisfare. Possono essere utilizzati come fonti nutritive per lo svolgimento della fermentazione, come agenti stabilizzanti e per migliorare alcune caratteristiche sensoriali dei vini. Questo progetto di dottorato mira a trovare le possibili relazioni tra la composizione chimica dei YDP e la loro funzione. Diversi prodotti commerciali verranno caratterizzati e testati come nutrienti per le fermentazioni, come agenti stabilizzanti e come antiossidanti per l'invecchiamento dei vini. Questo progetto potrà fornire importanti informazioni ai produttori di YDP che possono migliorare i loro metodi di produzione e la precisione dei loro prodotti. In questo modo, gli enologi potranno usufruire di YDP più specifici ed efficaci.

1. State-of-the-Art

Yeast derivative products (YDPs) are widely used in winemaking. In particular, YDPs can be used to enhance the fermentation processes (Pozo-Bayón *et al.,* 2009), to promote the tartaric and protein stability (Rigou *et al.,* 2021), to improve the antioxidant capacity (Lambert-Royo *et al.,* 2022), and to ameliorate the sensory characteristics of wines (Ruipérez *et al.,* 2022). Nowadays, the International Organization of Vine and Wine (OIV) has approved different typologies of YDPs, including inactivated yeasts, yeast autolysates, yeast protein extracts, yeast hulls and mannoproteins. They are used in different steps of winemaking depending on their composition and, consequently, their oenological ability. These products can be considered as an effective alternative to the use of chemicals, such as sulfur dioxide or inorganic nitrogen sources, implementing the sustainable production of wine.
Despite the importance of these products, the action mechanisms and the possible correlation between the chemical composition and the technological characteristics of YDPs have never been investigated in deep.
The main goal of this PhD project is the selection of YDPs with specific functions for the further improvement of precision oenology. This goal will be achieved by means of investigating the use of YDPs for specific applications and their mechanisms of action. Therefore, the specific objectives of the WInnY proposal will concern the use of YDPs as (i) nutrient source for both alcoholic [AF] and malolactic fermentations [MLF], (ii) stabilizing agent and (iii) antioxidant considering their impact on the overall composition of wine. Moreover, (iv) a crossover activity regarding a deep and novel characterization of YDPs will be performed in order to evidence, for the first time, a possible correlation existing between their composition and their specific ability.
The expected increase of knowledge will regard the composition and mechanisms of action of YDPs, in order to improve the accuracy of commercial YDPs and to provide directions to winemakers on their properties. These recommendations can also support the YDP producers being able to adequately setting up their production methods allowing to obtain YDPs with more specific functions and improved performances.

2. PhD Thesis Objectives and Milestones

In order to achieve the objectives described above, this PhD project is organized into 4 activities which are in turn divided into sub-activities, according to the Gantt chart reported in Table 1:

A1) **Characterization of YDPs.** Different commercial YDPs (20-25 products) will be characterized in terms of glutathione, cysteine residues (by UPLC-UV prior derivatization with *p*-benzoquinone), total nitrogen content (by enzymatic assays), amino acid profile (by UPLC-FL prior derivatization with *o*-phthalaldehyde) and antioxidant capacity (e.g., DPPH, ABTS, ORAC assays) (A1.1). Advanced characterization techniques such as spectroscopy, calorimetry and relaxometry NMR, will be used to determine the structure of yeast cell walls and their evolution with changes in the hydration level (A1.2).

It is expected to verify the composition differences between the YDPs and to build up a protocol for the application of the advanced techniques on YDPs.
Milestones: M1.1 – Complete chemical characterization of YDPs (month 9); M1.2 – Complete characterization of cell wall structure (month 17).

A2) **YDPs as source of nutrients for the fermentations.** YDPs will be used as source of nutrients for both AF (A2.1) and MLF (A2.2). AF trials will be carried out in laboratory scale using 2-4 different musts as medium. The fermentation trend will be monitored as well as the general chemical parameters (residual sugars, ethanol, pH, titrable acidity, organic acid profile, amino acid profile, aroma profile). MLF trials will be carried out in red wines. The concentrations of malic and lactic acids will be regularly determined (e.g., once a week) for monitoring MLF proceeding. Once MLF will be completed, the chemical parameters, the phenolic compounds and the biogenic amines will be determined.
It is expected to build up a link between their ability as fermentation enhancers and their chemical composition.

A3) **Improvement of wine stabilization in a sustainable perspective.** A large number of commercial YDPs (20-25 products) will be screened for tartaric and color stabilization in different wines (A3.1). The YDPs with the best performances will be added at variable amounts in 5-8 wines with different phenolic composition. The impact on tartaric stabilization and color as well as on phenols will be assessed (A3.2). In addition, the interactions between YDPs and phenols will be evaluated by using advanced techniques, as calorimetry and relaxometry NMR (A3.3).
It is expected to verify the effectiveness of YDPs as stabilizing agents, to select YDPs suitable for wine stabilization and to assess the relation between the YDP composition and the stabilizing activity.
Milestones: M3.1 - Complete screening of YDPs with stabilizing ability (month 6).

A4) **Exploitation of YDPs for wine aging.** In-bottle fermentation trials will be carried out with 2 white base wines following the *Champenoise* method. YDPs will be added before the fermentations and the sampling will be performed every 6 moths up to 18 months (A4.1). The samples will be subjected to accelerated aging trials (storage at 40 °C for 2 months in the dark) (A4.2). The antioxidant capacity, glutathione, cysteine residues, aromatic compounds (GC-MS) and sensory characteristics (e.g., mapping, quantitative-descriptive analysis) will be determined in the experimental wines.
YDPs are expected to effectively prolong the wine shelf life and enhance the sensory characteristics of the final product.

A5) **Bibliographic research, writing & editing:** PhD thesis, scientific papers, oral and/or poster communication for the dissemination of the results obtained from the WInnY project.

Table 1 Gantt chart for the WInnY project:

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Characterization of YDPs***																								
	1) Chemical characterization of YDPs																								
	2) Advanced characterization techniques																								
A2)	***YDPs as source of nutrients***																								
	1) Alcoholic fermentations																								
	2) Malolactic fermentations																								
A3)	***Improvement of wine stabilization***																								
	1) Screening of YDPs																								
	2) Selection of YDPs																								
	3) Exploitation of interactions with phenols																								
A4)	***Exploitation of YDPs for wine aging***																								
	1) In-bottle fermentation trials																								
	2) Accelerated aging trials																								
A5)	***Thesis and Paper writing and editing***																								

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Valorisation of Alternative Protein Sources by Tailored Biotechnological Processes and Non-Thermal Technologies to Obtain New Ingredients to Be Used in the Formulation of Innovative Foods

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Co-tutors: Prof. Francesca Patrignani, Dr. Davide Gottardi

This PhD thesis research project is aimed at obtaining new innovative ingredients from alternative protein sources using tailor made biotechnological processes and non-thermal technologies. After a careful selection of QPS microorganisms and matrices, the ingredients produced will be characterized and used to formulate innovative foods.

Valorizzazione di nuove fonti proteiche, attraverso processi biotecnologici di precisione e tecnologie non termiche, per l'ottenimento di ingredienti da utilizzare nella formulazione di alimenti innovativi

Questo progetto di tesi di dottorato mira all'ottenimento di nuovi ingredienti a partire da fonti proteiche alternative sfruttando processi biotecnologici e trattamenti non termici. Dopo un'attenta selezione di microrganismi con status QPS e delle matrici, gli ingredienti prodotti verranno caratterizzati e utilizzati per la formulazione di alimenti innovativi.

1. State-of-the-Art

Due to the progressive world population increase, it is necessary to find new sources of food and develop new techniques for valorising existing resources. In 2021, world consumption of protein of animal origin stood at around 478 million tons, with differences that mainly depend on the geographical area, traditions and prices. According to FAO, the quantities of protein sources of animal origin produced in the world will not increase in the next ten years and the quantities consumed per capita are expected to remain almost constant. However, market demand will increase, mainly due to the increase in the population that needs these resources (FAO, 2021). Therefore, obtaining and valorising proteins derived from alternative sources is arousing more and more interest, both from industry and from research. Examples of new protein sources can be obtained from vegetables such as legumes, cereals and pseudocereals or waste and vegetable by-products from the food and feed industry (Molfetta *et al*., 2022). These ingredients are suitably functionalized using tailored biotechnological approaches based on microorganisms for which the European Food Safety Authority (EFSA) has recognized the qualified presumption of safety (QPS) and identified as GRAS (Generally Recognized as Safe) by the Food and Drug Administration (FDA), or non-thermal processes such as high homogenization pressures (HPH) and pulsed electric fields that allow the efficient extraction and valorisation of high-quality proteins. For example, bacteria belonging to the genus *Bacillus* have been used to valorise vegetable waste to obtain bioactive compounds; yeasts such as *Saccharomyces cerevisiae* and lactic acid bacteria have been used to ferment waste from the processing industry of cereals, fruit, vegetables and legumes, significantly increasing the amount of functional peptides in the extracted products. Recently many studies are also focusing on the use of unconventional yeasts such as *Debaryomyces* spp., *Kluyveromyces marxianus* and *Yarrowia lipolytica* to valorise waste and by-products of the agri-food industry (Gottardi *et al*., 2021). For example, *Y. lipolytica* has been used to valorise another alternative protein source such as insect meals (Molfetta *et al*., 2022). In fact, following the growth of this yeast on cricket flour, protein hydrolysates were obtained, with increased functionality and higher protein/peptide content, also used as ingredients in the production of bakery products (Patrignani *et al*., 2020; Rossi *et al*., 2022; Rossi *et al*., 2021). In general, biotechnological processes make it possible to obtain compounds starting from waste and by-products and from alternative protein sources that demonstrate better antioxidant, antihypertensive, antimicrobial, preservative and aromatic activity compared to those obtained with other enhancement techniques. Therefore, suitably functionalized proteins and by-products can be reused in traditional and/or innovative food formulations, in line with today's objectives of sustainability and circular economy. EFSA has already recognized the safety of some alternative proteins deriving from vegetable sources and fermented vegetable waste and by-products, identified as novel foods pursuant to regulation (EU) 2015/2283 and usable as innovative ingredients in food products.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Bibliographic research** inherent to the topic, functional for the identification of the starting matrices (A1.2) and potentially exploitable microorganisms (A1.1), including unconventional yeasts, Bacilli and lactic acid bacteria.

A2) **Characterization of the identified strains and optimization of microbial performance** through the evaluation of the technological, functional and safety characteristics of the microorganisms (A2.1) in order to choose the most interesting for their enzymatic and metabolic activities.

A3) **Characterization of the matrices of interest** from a microbiological, nutritional and safety point of view (A3.1).

A4) **Obtaining and characterization of the ingredients** starting from the selected matrices and the use of the best performing microorganisms: identification of the most appropriate process conditions for the development of biotechnological processes on the selected matrices (as they are or non-thermally treated) (A4.1), characterization of innovative ingredients from safety, nutritional value and stability point of view (A4.2), their regulatory framework as established by EFSA (A4.3) and development of tailor-made protocols for their large-scale production.

A5) **Development of traditional or innovative products** using the most promising ingredients (A5.1) and characterization to evaluate their safety, microbiological shelf-life, quality, nutritional value and functionality (A5.2).

A6) **Writing and publication of the doctoral thesis, posters, scientific articles and oral presentation** (A6).

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1)	***Bibliographic research inherent to the topic***																		
	1) Alternative protein sources identification																		
	2) Safe and QPS microorganisms identification																		
A2)	***Characterization of strains and optimization of microbial performance***																		
	1) Evaluation of the technological, functional and safety characteristics of selected microbial strains, microbial strains selection and fermentative and technological performance optimization																		
A3)	***Characterization of the matrices of interest***																		
	1) Evaluation of microbiological, nutritional and safety characteristics of selected main protein sources																		
A4)	***Obtaining and characterization of ingredients***																		
	1)Preparation of biotechnological processes and optimization of process conditions (time, T, inoculum level)																		
	2) Evaluation of nutritional, stability and safety characteristics of obtained ingredients																		
	3) Regulatory framework																		
A5)	***Development of traditional or innovative products***																		
	1) Traditional/innovative food formulation including previously selected ingredients																		
	2) Characterization about safety, microbiological shelf-life, quality, nutritional value and functional aspects																		
A6)	***Thesis and articles preparation and participation in conferences***																		

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Use of Non-Thermal Treatments to Improve the Safety, Quality, and Shelf-life of Products of Animal Origin

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This PhD thesis research project is aimed at improving the safety, shelf-life and quality of animal foods, such as fresh sausages and dairy products, using non-thermal treatments including high-pressure processing (HPP), bioprotective cultures and natural antimicrobial compounds (i.e. essential oils).

Utilizzo di trattamenti non termici per il miglioramento della sicurezza, della qualità e della shelf-life di prodotti di origine animale

Questo progetto di tesi di dottorato mira a migliorare la sicurezza, la shelf-life e la qualità di prodotti di origine animale, come, ad esempio, salsicce fresche e prodotti lattiero caseari, utilizzando trattamenti non termici tra cui le alte pressioni idrostatiche (HPP), le colture di biocontrollo e l'utilizzo di composti antimicrobici naturali come gli oli essenziali.

1. State-of-the-Art

The market requires more and more minimally processed foods, less treated to safeguard the perceived safety, nutritional quality, and organoleptic aspects. In fact, the heat treatments applied to food cause chemico-physical, nutritional, and sensory changes that can reduce the quality of the product. For this reason, the goal of the processing technologies is to be mild for foods especially with respect to their nutritional value while diminishing any pathogenic and spoilage risk or any quality deterioration (*Valdramidis & Koutsoumanis*, 2016). To obtain a safe food matrix, the most important challenge is to avoid the presence of pathogenic microorganisms. Among them, Salmonella spp., *Listeria monocytogenes* and *Escherichia coli* are the most involved pathogens in foodborne outbreaks, while *Pseudomonas aeruginosa*, *Staphylococcus sp*., *Brochothrix thermosphacta*, *Psychrobacter sp*., *Micrococcus* sp., lactic acid bacteria and several strains of *Enterobacteriaceae* are the most reported microorganisms in the spoilage process (*Rosario et al*., 2021). The high-pressure processing is proposed as one of the non-thermal technologies for the inactivation of microorganisms and the prolongation of the shelf-life of foods. During HPP process, the matrix is subjected to pressures from 100 to 1,000 MPa in an aqueous medium at room temperature. The treatment can improve the microbiological quality of the products, promoting microbial inactivation by minimizing nutritional and sensory changes of the matrix as commercial applications generate a non-significant increase in the temperature of the treated food (*Aymerich et al*., 2008).
In addition to the application of non-thermal technologies for the treatment of products of animal origin, other strategies can be adopted, such as the application of natural antimicrobials (essential oils or their components, phenolic extracts, etc.), the use of bioprotective cultures and metabolites produced by them (bacteriocins). Consumers are aware of the negative health effects of food additives, and, for this reason, processed meat products obtained without adding chemicals are becoming increasingly important (*Balciunas et al*., 2013). In example, nitrates and nitrites can be reduced or replaced using natural substances present in spices, herbs, or essential oils, or deriving from microbial (bacteriocins) or animal (lysozyme) sources (*Oliveira et al*., 2018). However, the microorganisms used as bio-protection agents in foods have to show a high antagonistic activity against pathogenic and/or degrading microorganisms, must be safe for human health and must not have negative repercussions on the sensory and nutritional quality of the food product (*Oliveira et al*., 2018).
In this regard, my PhD research project is aimed at evaluating the application of the most interesting of these strategies to improve the organoleptic and nutritional quality and to prolong the shelf-life of products of animal origin (fresh sausage, dairy products) and assuring food safety avoiding the proliferation of pathogenic or toxin-producing microorganisms.

2. PhD Thesis Objectives and Milestones

The present research project aims to evaluate the use of non-thermal treatments to improve the safety, quality, and shelf-life of processed products of animal origin, in particular fresh pork sausages, and dairy products.

The PhD thesis project can be divided into the following activities, summarized in the Gantt chart shown in Table 1:
A1) **Bibliographic research** of non-thermal treatments on characteristics of processed foods of animal origin.
A2) **HPP application on fresh pork sausages** to evaluate its impact on meat color and microbial growth.
A3) **HPP application on dairy products** to evaluate their safety and shelf-life prolongation.
A4) **Use of plant extracts and/or essential oils** for the safety and quality enhancement of product of animal origin. Plant extracts and/or essential oils will be characterized as regard their composition. Their antimicrobial effects will be studied *in vitro* against common foodborne pathogens. The most interesting matrices will be tested in animal food models.
A5) **Use of bioprotective cultures** to increase the safety and quality of product of animal origin. Some bioprotective lactic acid bacteria strains will be tested for their antimicrobial activity *in vitro* and in animal food models. In addition, the cell free supernatants and/or purified antimicrobial peptides will be studied in the same matrices.
A6) **Writing and publication** of the doctoral thesis, posters, scientific articles, and oral presentation.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Bibliographic research***																								
A2)	***HPP application on fresh pork sausages***																								
	1) Evaluation of color by colorimeter and pigment analysis																								
	2) Microbiological analysis and evaluation of shelf-life																								
A3)	***HPP application on dairy products***																								
	1) Evaluation of product safety																								
	2) Prolongation of product shelf-life																								
A4)	***Study of plant extracts and/or essential oils***																								
	1) Study of the composition of plant extracts and/or essential oils																								
	2) Antimicrobial effects of plant extracts and/or essential oils																								
	3) Food model trials																								
A5)	***Study of bioprotective cultures***																								
	1) Cultures antimicrobial activity *in vitro*																								
	2) Cell free supernatants and/or purified antimicrobial peptides antimicrobial activity																								
	3) Food model trials																								
A6)	***Preparation of manuscripts, presentations, and thesis***																								

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Biotechnological approaches for the valorisation of by-products from marginal areas

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This PhD thesis research project aims to valorise waste, by-products and agro-industrial surpluses particularly abundant in marginal areas. The application of biotechnological processes, through the use of selected autochthonous microorganisms and/or coming from chemically similar matrices, will aim to increase the content of bioactive compounds present in the waste/by-product in order to produce functional foods and/or food supplements.

Approcci biotecnologici per la valorizzazione di sottoprodotti delle aree marginali

Questo progetto di tesi di dottorato mira a valorizzare scarti, sottoprodotti e surplus agro-industriali particolarmente abbondanti nelle aree marginali. L'applicazione di processi biotecnologici, attraverso l'uso di microrganismi selezionati autoctoni e/o provenienti da matrici chimicamente simili, avrà lo scopo di aumentare il contenuto di composti bioattivi presenti nello scarto/sottoprodotto al fine di produrre alimenti funzionali e/o integratori alimentari.

1. State-of-the-Art

In recent years, the necessity of sustainable development due to environmental pollution and a continuous decrease in natural resources has become increasing (Martins de Olivera M.et al., 2021). Moreover, food security currently appears to be seriously threatened by an exponential demographic development that requires alternative food sources without exhausting the agricultural sector. To address this issue, the use of by-products as ingredients in food could be a possible strategy (Barreira J. et al., 2019).
This PhD-project aims to valorise waste, by-products and/or agro-industrial surpluses from marginal areas (like almond, asparagus, artichoke etc.), by applying technological and bio-technological approached to enhance the bioavailability of functional compounds. In detail, at the first literature research focused on waste from the almond processing industry. Firstly, the almond tree is present in the marginal areas as it resists to high temperatures, to drought conditions and in the presence of poor soils (Romero et al., 2004). Secondly, almond processing generates large quantities of waste, such as hull, shell and skin, which are not used in the food sector but are mainly used as feed for livestock or as fuel (Garcia-Perez P. et al., 2021). However, the food industries show greater interest in these by-products as it is enriched in bioactive compounds, such as polyphenols or unsaturated fatty acids (Barral-Martinez M.et al., 2021). In addition, by-products that are generated by the extraction of almond oil (almond press-cake) are particularly rich in proteins. The treatment of proteolytic enzymes of almond press-cake can lead to the production of bioactive peptides (de Souza et al., 2020). In a recent study by Pasqualone et al. (2020) almond skin was used to increase the nutritional and functional value of biscuits. However, there are no studies in the literature that investigated the application of fermentation process on almonds by-products. The application of fermentation processes could be a strategy to further enrich the waste into bioactive compounds (Sadh et al., 2018) or to make them more bioaccessible to produce fortified foods and/or food supplements.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram showed in Table 1:

A1) **Chemical and microbiological characterisation of processing by-products, waste and/or agro-industrial surpluses**: microbiological characterisation by colture-dependent methods.

A2) **Isolation and selection of Lactic Acid Bacteria and optimization of fermentation protocol:** isolation of "autochthonous" LAB starting from the same matrix or after a spontaneous fermentation process of waste/by-products/agro-industrial surpluses; LAB selection with strong fermentative (growth and acidification curve) and proteolytic activity. Development and optimization of the fermentation process by modulating temperature, duration and percentage of inoculum.

A3) **Characterisation of fermented by-products/waste/agro-industrial surpluses:** chemical and microbiological characterization and evaluation of health-promoting activities through faecal batches.

A4) **Production of fortified food with processed by-products/waste/agro-industrial surpluses.**
A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 *Gantt diagram for this PhD thesis project.*

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A0)	***Literature search***																								
A1)	***Chemical and microbiological characterization***																								
A2)	***Isolation and selection of Lactic Acid Bacteria and development of the fermentation protocol***																								
A3)	***Characterization of fermented waste/by-products/agro-industrial surpluses***																								
A4)	***Production of fortified food***																								
A5)	***Thesis and Paper Preparation***																								

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Exploring drivers and barriers to the consumption of new plant-based foods in different population targets

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This PhD project is part of the ON Foods project (PNRR M4C2 – PE10: Models for sustainable nutrition) and aims to explore sensory quality of and consumer responses to new plant-based substitutes for animal products (e.g., meat, dairy and fish analogues), in order to provide a better understanding of drivers and barriers to their consumption and foster the transition toward a healthy and sustainable diet also considering the needs of vulnerable population targets, such as children and elderly people.

Indagine dei fattori che promuovono e ostacolano il consumo di nuovi alimenti a base vegetale in diversi target di popolazione

Questo progetto di dottorato si inserisce nell'ambito del progetto "ON Foods" (PNRR M4C2 – PE10: Modelli per una alimentazione sostenibile) e intende indagare la qualità sensoriale e la percezione dei consumatori verso nuovi alimenti a base vegetale, sviluppati come prodotti alternativi a quelli di origine animale (e.g. carne, prodotti lattiero-caseari e pesce vegetali), al fine di fornire una migliore comprensione dei fattori che ne promuovono o ostacolano il consumo favorendo la transizione verso una dieta sana e sostenibile, prendendo in considerazione anche le esigenze di gruppi vulnerabili della popolazione, come bambini e anziani.

1. State-of-the-Art

Nowadays, the food system is facing an ever-increasing demand for food due to population growth (Gibbs and Cappuccio, 2022). At the same time, the natural resources of land, water and energy are limited. Moreover, current food diets, rich in meat and energy-rich foods and poor in whole grains, fruit and vegetables, are no longer sustainable and have serious consequences for human health (Gibbs and Cappuccio, 2022). All these challenges can be addressed and overcome not only by applying more sustainable food production techniques, but also by the transition toward sustainable diets. For this reason, a major global need is to find alternative food sources with low environmental impact to meet the growing demand for food.

Promising solutions that have been receiving increasing attention in recent years are plant-based substitutes for animal food. These products attempt to mimic the appearance, smell, taste and texture of their conventional animal counterparts, such as meat, fish, milk and eggs (Alcorta *et al.*, 2021). This food sector includes plant-based meat analogues (i.e., sausages, chicken, burger, nuggets, tenders and cutlets), dairy alternatives (i.e., yoghurt, cheese, milk), egg substitutes (mayonnaise) and plant-based seafood (slices, fillets, fish sticks and fish burgers) (Alcorta *et al*., 2021). Being derived from botanical sources, such as legumes, seeds and nuts, pseudocereals, cereals and/or mushrooms (Tachie *et al.*, 2023), these foods are environmentally sustainable in terms of greenhouse gas emissions, land, water and energy use (Bryant, 2022). In addition, even if their long-term impact on health is still uncertain (Tso and Forde, 2021), they are lower in fat and cholesterol and higher in fibre than animal origin products, contributing to lowering the risk of cardiovascular disease (Bryant, 2022).

In Europe, the plant-based sector has grown of 22% compared to 2020 (GFI, 2022), showing consumers' growing interest in alternative protein sources. However, this sector remains a niche market since their consumption is still hampered by several obstacles. In general, drivers and barriers to healthy and sustainable food can be classified into person-related factors such as socio-demographic, dietary status, psychological and physiological variables, as well as product-related factors, including food convenience (e.g., price, preparation time, food availability), credence attributes (e.g., healthiness, naturalness, sustainability and animal welfare) and sensory properties (Giacalone *et al.*, 2022). Indeed, the exploitation of plant-based proteins pose several challenges about the sensory characteristics of the products, being very difficult to replicate the appearance, taste, flavour and texture of animal origin food. For example, these products are often characterised by beany flavour and bitter taste or astringency (Fiorentini *et al.*, 2020) and have textural properties that differ significantly from those of animal origin, disregarding consumers' expectations (Alcorta *et al.*, 2021). Since food consumption is mainly driven by preferences, to foster the development of new plant-based foods, it is necessary to optimize their sensory characteristics to mimic those of the original animal version. In this context, sensory studies and consumer science provide valuable support in understanding how consumers perceive food and which sensory attributes should be modulated to increase their acceptance. Developing new food products that are not only healthy and sustainable, but also acceptable to the consumer is crucial to drive the transition towards sustainable food chains.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:
This PhD project is part of the PNRR project "ON Foods - Research and innovation network on food and nutrition Sustainability, Safety and Security - Working ON Foods", aimed at improving the future of the community and the supply chain through a new sustainable food model (www.onfoods.it).
The overall aim of this PhD project is to explore sensory quality of and consumer responses to new plant-based food alternatives to conventional animal counterparts (e.g., meat, dairy, eggs and fish). In particular, the perceptive and behavioural determinants that can positively or negatively influence consumer liking of plant-based food alternatives will be investigated also in vulnerable population targets (children and elderly people).
The main activities of these three years will be organized according to the Gantt diagram given in Figure 1:
First, a literature review on plant-based food alternatives will be performed in order to formulate the research strategy and develop the overall research design. Literature review will be conducted in parallel with an online Italian market inventory to create a database of animal-origin analogues. For each food, easily available information from the packaging will be collected (e.g., composition, price, specific slogans). Based on literature gaps and according to criteria such as nutritional composition and representativeness of the Italian market, specific plant-based food categories and products will be selected for sensory and consumer testing with different population targets. The participant's acceptance of selected food products will be investigated using hedonic methods, while their sensory attributes will be measured through consumer-friendly descriptive approaches (e.g. Check-All-That-Apply, CATA). Information about participants' socio-demographics, food consumption behaviour, and personality traits (e.g., food neophobia) will be collected together with emotions while consuming such products. In order to assess the cross-national differences in the determinants of plant-based analogues acceptance, a part of the PhD project will be performed abroad.

Figure 1 Gantt diagram for this PhD thesis project.

ACTIVITIES	YEAR 1												YEAR 2												YEAR 3											
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Literature review																																				
Formulate research strategy, research design, select methods																																				
Market inventory and creation of a database of plant-based foods																																				
Selection of a subset of products representative of the market																																				
Main test: selected plant-based products with adults consumers																																				
Main test: selected plant-based products with children																																				
Main test: selected plant-based products with elderly people																																				
Cross-national study (study set-up and experience abroad)																																				
Data analysis																																				
Manuscripts preparations																																				
Thesis preparation																																				

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Tailoring qualitative properties of foods by technology: use of innovative technologies for improving safety, quality and sustainability

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This PhD thesis research project is aimed to develop innovative food products through the investigation and application of innovative technologies such as pulsed electric field (PEF) and high pressure processing (HPP). Furthermore, to improve several qualitative and quantitative aspects of food such as safety, sustainability as well as sensorial, functional and nutritional quality of cereals and legumes-based products. Finally, the effect of the non-conventional processes will be compared with conventional processes.

Approcci tecnologici per progettare la qualità degli alimenti: utilizzo di tecnologie innovative per migliorare sicurezza, qualità e sostenibilità

Questo progetto di tesi di dottorato ha lo scopo di sviluppare prodotti alimentari innovativi attraverso lo studio e l'applicazione di tecnologie come i campi elettrici pulsati (PEF) e i trattamenti ad alta pressione (HPP). Inoltre, si ha l'obiettivo di migliorare diversi aspetti qualitativi e quantitativi come la sicurezza, la sostenibilità e la qualità sensoriale, funzionale e nutrizionale dei prodotti a base di cereali e legumi. Infine, verrà confrontata l'applicazione di trattamenti convenzionali con trattamenti non convenzionali.

1. State of the Art

In recent years, many novel technologies of food processing have evolved in response to the need of consumers for safe and high-quality food products (Niu et al., 2020). Nowadays, consumers increasingly demand a large variety of high-quality and show interest in food of high nutritional value and organoleptic properties for a healthy product. In addition, the bakery and pasta sectors have received a large request for products delivering health benefits and/or specifically designed for particular groups of consumers (e.g. celiac, diabetic, etc.) (Guiné et al., 2020). For this reason, the food industry must constantly innovate and launch new products to respond to consumer demand.

Moreover, ancient varieties of cereals and legumes are valuable sources of essential nutrients and bioactive compounds, the incorporation of these ancient varieties will be important in food industries due to their nutritional values compared to the modern varieties (Zamaratskaia, Gerhardt and Wendin, 2021). Presently, an interest in the ancient varieties of wheat has been established as a healthier nutritional profile than modern kinds of wheat. In addition, several studies on humans result in an improvement in pro-inflammatory/antioxidant parameters, and glycemic and lipid status with the consumption of ancient wheat varieties, however, the mechanisms responsible for these beneficial effects are not completely understood (Dinu et al., 2018). This information raises an interesting future for the ancient varieties by studying their chemical composition and functional properties.

Therefore, this project aims to improve the qualitative and quantitative attributes of plant-based products by modifying the bio-macromolecules using novel and minimal processing technologies. These novel technologies include but are not limited to the use of Pulsed Electric Field (PEF) and High-Pressure Processing (HPP) combined with fermentation to ensure the quality and safety of foods while maintaining environmental sustainability throughout the shelf life of foods. The second objective of this project is to optimize the processing parameters using experimental design and Response Surface Methodology.

ONFOODS (Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.3 - Call for tender No. 341 of 15 March 2022 of the Italian Ministry of University and Research funded by the European Union - NextGenerationEU) has a strong focus on the improvement of food quality and nutrition to meet the needs and expectations of modern consumers.

2. PhD Thesis Objectives and Milestones

According to the Gantt chart (Table 1), this PhD research project can be divided into the following activities within the general objective described above:

A1) **Cereals and legumes characterization:** Selection of at least 3 cereals and 3 legumes based on their functional properties and characterization of ancient varieties of cereals and legumes flour through the following analyses: rheological behaviour, Differential Scanning Calorimetry (DSC), LR NMR (A1.1), starch analysis, texture and colour analyses, antioxidant capacity (A1.2).

A2) **Application of conventional processes:** application of thermal process as a pre-gelatinization of starch in flour from ancient varieties of cereals/legumes (A2.1). Moreover, the thermally treated flour will be fermented (A2.2) to evaluate the effect of thermal treatment on the microbial growth.

A3) **Application of non-conventional processes:** application of PEF and HPP on the flour from ancient varieties of cereals/legumes to improve the techno-functional properties. Improvement of the fermentation behaviour especially the microbial growth by creating a system and optimizing the processing parameters. Improvement of the extraction yield of bioactive compounds from cereals/legumes flour with PEF (A3.1). Finally, an improvement of the fermentation behaviour after HPP treatment of the starch pre-gelatinization by optimizing the processing parameters (A3.2).
Comparison of the conventional and non-conventional processes to evaluate the differences between the treatments in the microbial growth, the accessibility of nutrients for the microorganism, and techno-functional properties.

A4) **Development of high-quality products:** formulation of nutritious and high-quality products (gluten-free bread, crackers) for specific consumer group (A4.1) and shelf-life evaluation (bread staling) through the following analyses: microbial load, sensory analyses, resistant starch, texture and colour analyses (A4.2).

A5) **Writing and editing of the PhD thesis** and publication of scientific papers.

Expected results:

1. Optimized processing parameters of the conventional and non-conventional processes for the appropriate modification of the bio-macromolecules, the techno-functional properties, and improvement of the microbial growth.
2. Optimized processing steps for increased shelf-life of the product (bread, crackers), and to improve the sensory acceptability.
3. Optimized products for specific consumer group.

Table 1: Gantt diagram of the PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Cereals and legumes characterization***																								
	1) Chemical composition																								
	2) Functional properties																								
A2)	***Conventional technologies***																								
	1) Thermal treatment																								
	2) Fermentation																								
A3)	***Non-conventional technologies***																								
	1) PEF treatment																								
	2) HPP treatment																								
A4)	***Development of high-quality products***																								
	1) Formulation and optimization																								
	2) Shelf-life evaluation																								
A5)	***Thesis and Paper Preparation***																								

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Bioactive-Rich Mushrooms for Food Reformulation (BIOMUSH-FOOD)

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This PhD research project is aimed at the development and optimisation of foods enriched with bioactive-rich mushrooms (BRMs) by applying DoE techniques and RSM. After the optimization of BRMs cultivation conditions, the obtained biomasses will be included in different food products. In particular, BRMs integration will be optimized for milk- and plant-based desserts as well as for traditional and gluten-free baked products. The enriched foods will be studied for quality characteristics, including sensory properties, and *in vitro* digestibility to evaluate the fate of bioactive compounds in digestate.

Funghi ricchi in composti bioattivi per la riformulazione di prodotti alimentari (BIOMUSH-FOOD)

Questo progetto di ricerca di dottorato è finalizzato allo sviluppo e all'ottimizzazione di alimenti arricchiti con funghi ricchi di composti bioattivi (BRMs) mediante l'applicazione di tecniche DoE e RSM. In seguito all'ottimizzazione delle condizioni di coltivazione dei BRMs, le biomasse ottenute saranno incluse in diversi prodotti alimentari. In particolare, l'integrazione con BRMs sarà ottimizzata per dessert a base latte e vegetale, nonché per prodotti da forno tradizionali e senza glutine. Gli alimenti arricchiti saranno valutati in termini di caratteristiche qualitative, incluse le proprietà sensoriali, e di digeribilità *in vitro*, al fine di indagare il destino dei composti bioattivi nel digestato.

1. State-of-the-Art

Global concerns around the consumption of animal products and their adverse effects on health and environment have led to significant growth in the plant-based protein field. Plants, like other natural products including mushrooms, have seen an increase in their production also due to their content in bioactive and health-promoting substances.

Bioactive-rich mushrooms (BRMs) can be defined as macroscopic fungi, mostly higher *Basidiomycetes*, which are used in the form of extracts or powder for prevention, alleviation, or healing of diseases and/or for nutritional reasons (Lindequist *et al*., 2014). Presently, BRMs are mainly used as dietary supplements or functional foods, especially in Eastern countries. The most important BRMs species are *Ganoderma lucidum, Lentinula edodes, Agaricus brasiliensis, Pleurotus ostreatus, Cordyceps sinensis, Grifola frondose*, and some others.

From a nutritional point of view, mushrooms are valuable health foods since they have a significant amount of dietary fiber and are poor in calories and fat (Roncero-Ramos *et al*., 2017). Moreover, they have a good protein content (20–30% of dry matter), which includes most of the essential amino acids, a nutritionally significant content of vitamins, and trace minerals. They contain bioactive compounds of high medicinal value such as lectins, polysaccharides, phenolics, and volatile organic compounds, which are considered as relevant responsible agents for healthy activities including antitumor, antioxidant, antihypercholesterolemia, and antidiabetic effects.

Besides nutritional composition of mushrooms, it could be interesting to evaluate their *in vitro* digestibility, especially to study whether bioactive compounds are available during digestion, which can have benefits on health. Different *in vitro* methods have been developed to simulate digestion processes, from the oral to the small intestinal phases and, occasionally, large intestinal fermentation, taking into account the digestive enzymes, their concentration, pH, and digestion time (Minekus *et al*., 2014). In particular, an international network of multidisciplinary experts harmonized the digestion conditions by publishing the INFOGEST 2.0 method (Brodkorb *et al*., 2019).

Thanks to the valuable characteristics, BRMs could be used to reformulate foods to enrich the nutritional profile and provide consumers with good alternative proteins. In the literature there are still few food applications of mushroom biomasses, mainly focused on bakery products (Li *et al*., 2008; Prodhan *et al*., 2015), thus asking for new investigations and for the health benefits validation in the final products. Lu *et al*. (2020) reported that novel applications for mycelia- and fruiting body-based macrofungal foods are being explored for the improvement of food flavor and nutrition. However, in some cases an extraction phase is necessary, which may negatively influence the structure and biological activity of the bioactive compounds.

2. PhD Thesis Objectives and Milestones

The overall objective of this PhD research project is to integrate BRMs, grown on agro-food wastes, in different

food formulations to improve their nutritional profile. To fulfil the overall objective, the PhD project will be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Literature survey about BRMs.** The different characteristics of BRMs will be studied, focusing, in particular, on the nutritional aspects and the bioactive compounds with health implications. Moreover, a survey about cultivation methods and conditions will be performed, to study the relationship with biomass properties.

A2) **Optimization of mushroom biomass cultivation conditions.** Mushrooms with the best technological and nutritional characteristics will be chosen to study cultivation performances both in solid and liquid state. BRMs will be grown on different agrifood wastes, applying different environmental parameters (i.e., temperature, humidity, organic components) to study the effect on the biomass characteristics and to optimize cultivation conditions. A further in-depth analysis of the biomass will involve the evaluation whether to use it as such or to proceed with the extraction of proteins and bioactive components.
Milestone (M) 1: Optimized cultivation conditions to obtain valuable BRMs biomasses for food applications.
Risk (R) 1: Difficulties to obtain a constant quality biomass. *Mitigation action*: introducing new parameters to control the BRMs growing or changing mushroom species.
R2: Difficulties to obtain dry biomasses that maintain the nutritional properties. *Mitigation action*: considering an extraction phase and/or use a milder preservation technology that respects the biomass values.

A3) **Food development and optimization.** Design of Experiment techniques (DoE) and Response Surface Methodology (RSM) will be used to find the best integration level of BRMs in terms of final product quality and to optimize food formulations. In particular, BRMs integration will be optimized for milk- and plant-based ice creams and mousses as well as for traditional and gluten-free bread and cookies. For each product, a comparison will be made considering reference food formulations. Each product will be analysed for quality attributes, including sensory features, and for nutritional properties. In particular, *in vitro* digestibility will be studied according to the INFOGEST protocol to verify the digestion fate and the bioaccessibility of the bioactive compounds.
M2: Optimized formulations of BRMs enriched foods.
R3: Difficulties in the design of foods with the chosen mushroom biomasses. *Mitigation action*: revision of the formulation and/or choice of different food products.

A6) **Data elaboration.** The most suitable statistical tools will be applied to all the collected data.

A7) **Manuscript preparation.** During the three-year project, scientific papers and oral/poster communications will be prepared, thus assuring proper dissemination of the results.

Table 1 Gantt diagram of the PhD thesis project

		Year 1												***Year 2***												***Year 3***											
Activity		1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
A1)	***Literature survey***																																				
A2)	***Optimization of BRMs cultivation conditions***																																				
A3)	***Food development and optimization***																																				
	Baked products																																				
	Desserts																																				
A6)	***Data elaboration***																																				
A7)	***Manuscript preparation***																																				

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Development of new approaches for the evaluation of the metabolism of bioactive compounds of nutritional interest

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This PhD thesis research project aims to apply desorption electrospray ionization (DESI) mass spectrometry imaging (MSI) to evaluate (poly)phenol distribution in tissues. This innovative analytical technique will allow us to shed light on the transport mechanisms and cellular absorption involved in the uptake of phenolic compounds. The objective is therefore to verify the relationship between ingested (poly)phenols, metabolite and colonic catabolite production and their ability to reach target organs in which they can exert their functionality.

Sviluppo di nuovi approcci per la valutazione del metabolismo di composti bioattivi di interesse nutrizionale

Questo progetto di ricerca di tesi di dottorato si propone di applicare la spettrometria di massa *imaging* (MSI) a desorbimento per ionizzazione elettrospray (DESI) per valutare la distribuzione dei (poli)fenoli nei tessuti. Questa tecnica analitica innovativa ci permetterà di far luce sui meccanismi di trasporto e assorbimento cellulare coinvolti nell'*uptake* dei composti fenolici. L'obiettivo è quindi verificare la relazione tra i (poli)fenoli ingeriti, la produzione di specifici metaboliti e cataboliti colonici e la loro abilità a raggiunge gli organi *target* nei quali possono svolgere la loro potenziale attività funzionale.

1. State-of-the-Art

(Poly)phenols are organic compounds produced by plants as secondary metabolites and are abundantly present in fruits, vegetables, nuts, herbs, and other plant-derived products (Del Rio *et al*., 2013). Various studies have demonstrated the potential positive impacts of these compounds on human health (Rana *et al*., 2022). Nonetheless, the comprehension of the absorption and distribution mechanisms of phenolic compounds within the human body is of great importance for precisely identifying their bioactive form in target organs or tissues and, consequently, for understanding their biological effects.
Mass spectrometry imaging, or MSI, is a highly effective analytical method that provides a visual *in situ* representation of the molecular distribution within complex samples and biological tissues. This technique allows to create a map of various molecules in a tissue sample, providing spatial information that cannot be obtained through traditional analytical techniques (Chughtai and Heeren, 2010). Desorption electrospray ionization (DESI) is an imaging source that, thanks to a charged solvent, ionizes the compounds directly on the tissue at atmospheric pressure and is ideal for the detection of small molecules that range from 50 to 2000 Da (Monge *et al*., 2013). The available literature shows that the application of MSI for the study of phenolic compounds is yet to get a foothold but is expected to increase as researchers seek to advance their comprehension of the health-promoting attributes of (poly)phenols and their metabolites. DESI MSI represents a viable approach for exploring the absorption and distribution of bioactive compounds in tissues, useful to enhance the comprehension of the pharmacokinetics of (poly)phenols and their metabolites, along with their possible site of action and transport mechanisms implicated in their distribution.

2. PhD Thesis Objectives and Milestones

During the 1st year of the PhD, the activities were focused on literature search to write one review on the applications of MSI so far published for evaluating (poly)phenol distribution in animal tissues, and a second review on the absorption and transport mechanisms involved in the distribution of (poly)phenols in target organs and tissues.

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities, according to the Gantt diagram given in Table 1:
A1) **Training with DESI MSI** to develop the method (A1.1), including verification of sensitivity, reproducibility, and functionality of the instrument, and preliminary **trials** (A1.2).
A2) ***In vivo* study** with 36 C57BL6/J male mice, giving the standard compound deuterated D5-(−)-epicatechin via oral gavage. The mice will be sacrificed in groups of 4 at different timepoints, and all the organs will be collected,

together with plasma, urine, and feces.
A3) **Analysis of collected samples**, including i) plasma, urine, and feces to check the nutrikinetics of the administered compound (A3.1); ii) microscopy evaluation of cross-sectional tissue/organ slices to acquire the histological image (A3.2); iii) study of the images obtained from DESI MSI for the *in situ* visualization of D5-(−)-epicatechin metabolites and catabolites to evaluate the *in vivo* distribution within animal's tissues and organs, with a focus first on the gastrointestinal tract, and subsequentially on the other organs (A3.3 and A3.4).
A4) **Writing and editing** the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Training with DESI MSI***																								
	1) Method development																								
	2) Preliminary trials																								
A2)	***In vivo study***																								
A3)	***Analysis of collected samples***																								
	1) Plasma, urine, and feces																								
	2) Histological evaluation																								
	3) Gastrointestinal tract DESI images																								
	4) Other organs DESI images																								
A4)	***Thesis and Paper Preparation***																								

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Innovative strategies to modulate Delivery and Food Structuring abilities of Starch-based Ingredients

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This PhD research project aims at applying an un-conventional physical technology, i.e. ball milling for the development of new starch ingredients with modified molecular properties and technological functionalities and their applications for food structuring and delivery systems. Different ball-milling process variables (rotation rate, time) will be tested on starches of different botanical origin (corn, potato, tapioca) and the corresponding chemical, physical, microstructural, and technological properties will be evaluated. Physically modified starches will be, then, used to design and develop delivery systems (e.g., emulsions, microencapsulates) and/or structures (cryogels) with new functionalities for innovative food systems applications.

Strategie innovative per modulare le capacità di consegna e di strutturazione degli alimenti degli ingredienti a base di amido

Questo progetto di ricerca di dottorato mira all'applicazione di una tecnologia fisica non convenzionale, ovvero la macinazione a sfere per lo sviluppo di nuovi ingredienti base amido con proprietà molecolari e funzionalità tecnologiche modificate e la loro applicazione come agenti strutturanti o in sistemi di rilascio. Verranno testate diverse variabili di processo della macinazione a sfere (velocità di rotazione, tempo) su amidi di diversa origine botanica (mais, patate, tapioca) e verranno valutate le corrispondenti proprietà chimiche, fisiche, microstrutturali e tecnologiche. Gli amidi modificati fisicamente saranno, quindi, utilizzati per progettare e sviluppare sistemi di stabilizzazione e rilascio (ad esempio emulsioni, microincapsulati) e/o strutture (criogel) con nuove funzionalità per applicazioni innovative in sistemi alimentari.

1. State-of-the-Art

Starch is a natural biopolymer found in cereals and many plants-based products. It is made of glucose units organised in a complex multi-scale structure resulting from the arrangement of amylose and amylopectin. There is an increasing demand and importance of starch in food applications due to its interesting properties that contribute to the appearance, structure, and quality of the products. However, in its native state, starch has certain limitations that restrict its use. These limitations include poor solubility in cold water and its inability to withstand harsh processing conditions (e.g. high temperature) long hydration and swelling time, and tendency towards retrogradation. To overcome these issues, technologies to modify it have been developed by using physical, chemical, enzymatic methods, or combinations thereof (Baranowska and Kowalczewski, 2022) that result in improved technological properties (e.g., solubility, water, and oil binding capacity). Physical technologies such as high pressure (HPP & HPH), cold plasma, pulsed electric field, and ball milling are being increasingly utilized for starch modification. These environmentally friendly techniques with wider consumer acceptance show great potential for producing ingredients with enhanced functionality in foods and allow manufacturer to avoid the "modified starch label designation". Ball milling as an emerging and "green" technology is used as a physical processing method to modify starch. Recent studies demonstrated for its ability to enhance the properties and functionalities of the processed materials (Bangar *et al.*, 2023). Some authors (Huang *et al.*, 2008; Juarez-Arellano *et al.*, 2021) have reported on the use of ball milling for starch modification and their studies revealed improved amylose content, greater cold-water solubility, and lower temperature of gelatinisation. In food industry various are the applications of starch, as both native or modified and among others, the ones growing interest relate to encapsulation and the development of gel-like structures. For instance, this biopolymer can form V-type inclusion complexes with tiny hydrophobic molecules (e.g., lipophilic vitamins, flavours, phenolics) within the inner helical cavity of amylose (Zhang *et al.*, 2023, Zhou and Kong, 2023). The growing use of "gel" structures in the food sector is driven by their advantages in terms of cost, availability, and digestibility. In fact, after gelatinisation and cooling, starch can reform an ordered structure (retrogradation) leading to the formation of three-dimensional network (hydrogel) with interesting properties and applications. Starch-based aerogels and cryogels, highly porous matrices obtained via starch dissolution and drying by supercritical CO_2 or freeze-drying respectively, are increasingly used for both food and non-food delivery purposes (Cuo *et al*., 2021, Zou & Budtova, 2021). Eventually, starch granules represent a natural nontoxic emulsifier for particle-stabilised emulsified systems (Pickering emulsions), characterised by excellent long-term stability against coalescence. In the field of starch-based materials, despite data available in the literature, a significant research gap still exists on the development

of starch-based structures, in particular the more innovative ones (gels), and the technological functionality of ball-milled starch, as well as their application for encapsulation of lipophilic and hydrophilic compounds by conventional and/or innovative (i.e. co-milling) purposes, along with their potential ability for stabilizing Pickering emulsions.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) **State-of-the art and methods of optimisation**. Literature review on starch, modifications by physical processes and technological functionality. Optimisation of methods for the evaluation of physical, microstructural properties and technological functionality (A1.1) (e.g., water solubility and swelling power, water and oil absorption test, microstructure, rheological and thermal behaviour).

A2) **Ball-milling (BM) starch modification:** Different process conditions on starch of different origin (corn, potato, and tapioca) will be applied: (time, rotation rate); processed BM starches will be characterised for morphological, physical (A2.1), technological functionality and stability (A2.2)

A3) **Design and development of BM-starch structures:** In this task, hydrogel and cryogel structures will be prepared and characterised. Hydrogels will be prepared by using selected BM-modified starch at different process time (5,15,30 min) and heated at 90 ºC. Their corresponding physical and functional characteristics will be determined (textural, thermal, crystallinity, granular morphology, retrogradation). Cryogels will be obtained by freeze-drying and characterized for density, porosity, moisture sorption isotherm, thermal properties, granular morphology, textural analysis, absorption capacity, crystallinity, mechanical properties.

A4) **Design and development of BM-based microencapsulates and structures as delivery systems**: Microencapsulated powders will be obtained by co-milling of mix of starch and lipophilic compounds and by Pickering emulsions and spray-drying. Complex gel structures (hydrogels, cryogels) will be developed by using BM-starches in presence of **other co-solutes and bio-actives of interest (e.g., oleuropeins, β-carotene)** Physical and microstructural properties along with digestibility and in-vitro bioavailability, release rate of the encapsulated selected compounds will be studied.

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***State-of the art and methods of optimisation***																								
	1) Physical and microstructural properties																								
	2) Technological functionality																								
A2)	***Ball-milling starch modification***																								
	1) Technological functionality																								
A3)	***Design and development of BM-starch structures***																								
A4)	***Design and development of BM-based microencapsulates and structures as delivery systems***																								
A5)	***Thesis and Paper Preparation***																								

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Study of Innovative and Sustainable Approaches for the Mitigation of Contamination with Mineral Oils in Vegetable Oils and Fats

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As the standard of living improves, concerns over food safety and potential contaminants will continue to be an important health issue. The aim of this PhD research project is to study the sustainable approaches for the mitigation of contamination with mineral oils in vegetable oils and fats. These compounds are associated with risks to human health especially as carcinogenic factors. To this purpose, methods capable of detecting carcinogenic hydrocarbons in selected mineral oils will be studied and optimised, then applied to assess the decontamination properties of conventional and innovative adsorbents.

Studio di approcci innovativi e sostenibili per la mitigazione della contaminazione da oli minerali in oli e grassi vegetali

Con il miglioramento del tenore di vita, le preoccupazioni per la sicurezza alimentare e i potenziali contaminanti continueranno a essere un importante problema sanitario. L'obiettivo di questo progetto di ricerca di dottorato è di studiare approcci sostenibili per la mitigazione della contaminazione da oli minerali negli oli e nei grassi vegetali. Questi composti sono associati a rischi per la salute umana in quanto cancerogeni. A tal fine, verranno studiati e ottimizzati metodi in grado di rilevare gli idrocarburi minerali cancerogeni selezionati, per poi essere applicati per valutare le proprietà decontaminanti di adsorbenti convenzionali e innovativi.

1. State-of-the-Art

Nowadays, food safety is a concern matter, especially when regarding the possible presence of genotoxic and carcinogenic compounds in food. Mineral oils mainly consist of saturated hydrocarbons (MOSH) able to bio-accumulate in human tissue and organs, and aromatic hydrocarbons (MOAH). MOAH contamination has become of public interest due to its harmful effects on human health. Polycyclic aromatic compounds with >3 rings non alkylated or of low alkylation degree are of high concern due to their recognized genotoxic and carcinogenic properties (EFSA, 2012; Jaén et al., 2022). Mineral oil hydrocarbon (MOH) contamination in foods such as vegetables oils and fats may occur at any stage of material production (Purcaro et al., 2016).
Partial MOH mitigation can be achieved by increasing the temperature during the deodorization step, but this can lead to the other side to the formation of other contaminants. It is well known that some absorbents such as activated carbons (ACs) used during the bleaching step of edible oils may play an important role in retaining parent polycyclic aromatic hydrocarbons (PAHs) (Torres et al., 2021), but their effectiveness against the most relevant genotoxic and carcinogenic fraction of MOH has not been evaluated to date. AC is a popular choice among all owing to its good adsorption capacity, active free valences, high surface area, porous structure, surface reactivity, inertness, and thermal stability (Soni et al., 2020). Furthermore, mesoporous silica nanoparticles (SiO_2 NPs) can be used also as adsorbent. SiO_2 NPs are the most used nowadays as adsorbent material for their attractive properties like stability, low toxicity, and the ability to be functionalized with various molecules and polymers. It also has various applications as an additive for rubber and plastic production (Ali 2016; Sadegh et al., 2017). Biological decontamination by microorganism has been widely described in aqueous environment (Kajla et al., 2021), but scarcely studied and applied on vegetable oils.
Several protocols for isolating the targeted MOAH (>3 rings and low degree of alkylation) from the oil matrix prior to analytical determination will be carried out. A set of protocols using online HPLC-GC-FID, GC-MS, GC×GC-FID/MS will be optimized and applied for determining selected polycyclic aromatic compounds (alkylated and not alkylated) chosen as model molecules, and for group-type separation of the MOAH. The developed protocols will be then applied to test decontamination properties of conventional adsorbent (bleaching earths and ACs) used for oil bleaching. New adsorbent materials such as SiO_2 NPs will be also studied, characterized, and optimized, which can be in terms of enhancing the shape, size, pores, and crystallinity properties to facilitate the decontamination efficacy against the targeted compounds. The surface area and the porosity of the synthesized NPs could be determined by nitrogen (N_2) gas adsorption-desorption analysis, while NPs properties by X-ray diffraction (XRD) for crystallinity, morphological studies, and size by field emission scanning electron microscopy (FESEM). Finally, biological decontamination using bio-surfactants able to solubilize these hydrophobic contaminants in an aqueous environment, favouring decontamination processes by microorganisms, could be tested. The most promising decontamination process will be scaled-up at Unigrà srl company.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Developing new protocols** for isolating and detecting genotoxic carcinogenic PAHs and MOAH (>3 rings and little alkylation) from edible oils.

A2) **Studying physicochemical properties and evaluating the behaviour** of adsorbent materials such as ACs, and SiO_2 NPs for removing contaminations.

A3) **Spending six months undertaking research abroad** in collaboration with other research teams. The main goal could be: 1) improve the analytical methodology, and/or 2) deepen the study on adsorbent properties, and/or 3) evaluate biological decontamination by microorganisms.

A4) **Spending six months at Unigrà srl** to scale-up the most promising decontamination method.

A5) **Data processing.**

A6) **Writing and Editing of the PhD thesis**, scientific papers and oral and/or poster communication.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
Literature review																			
A1)	***Developing new protocols for detection***																		
	1) Protocol comparison																		
	2) Protocol optimization																		
A2)	***Adsorbent characterization, optimization, and testing***																		
	1) Adsorbent properties																		
	2) Adsorbent optimization and testing																		
A3)	***Undertaking research abroad***																		
A4)	***Process scale-up at Unigrà srl***																		
A5)	***Data processing***																		
A6)	***Thesis and paper preparation***																		

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Development of High Nutritional Quality Food Ingredients from Avocado Production Wastes and By-products

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Considering the significant increase in tropical fruit production in Sicily, this PhD thesis project aims to develop high nutritional quality food ingredients from the wastes and by-products of avocado production. Defatted pulps, peels, leaves, and seeds will be used to produce flours, through different drying technologies, and their chemical, nutritional, and sensory quality and consumer acceptability will be evaluated. The flour obtained will be proposed as a food ingredient in meat and baked products.

Sviluppo di ingredienti alimentari di elevata qualità nutrizionale da scarti della produzione di avocado

Considerato il significativo aumento della produzione di frutta tropicale in Sicilia, questo progetto di tesi di dottorato mira a sviluppare ingredienti alimentari di elevata qualità nutrizionale da scarti e sottoprodotti della filiera di produzione dell'avocado. A tal fine le polpe disoleate, le bucce, le foglie e i semi di avocado verranno utilizzati per la produzione di farine attraverso differenti tecnologie di essiccamento e ne verranno valutate le caratteristiche chimiche, nutrizionali, sensoriali e l'accettabilità del consumatore. Le farine saranno, quindi, proposte per il loro utilizzo quali ingredienti in prodotti carnei e da forno.

1. State-of-the-Art

Avocado (*Persea americana* Mill.) is a tropical fruit native to Mexico and Central America, but nowadays widely produced and consumed worldwide. The market for processed avocados is projected to reach US $ 2.70 billion by 2024 with an increase of about 25% in the last five years (Nyakang'i *et al.,* 2023). The increased request for avocado, as raw and processed, leads to large amounts of by-products like seeds, peels, and defatted pulps which account for approximately 30% of fruit weight, which must be disposed of. At the same time, avocado by-products are rich sources of carbohydrates (seed 27.5-82%, peel 43-81%), lipids (seed 0.5-15%; peel 2-11.4%), proteins (seed 0.14-9%; peel 0.17-8%), vitamins such as ascorbic acid, vitamin E, polyphenols, and carotenoids whose amount greatly varies according to the variety and production area. Moreover, since avocado trees are subjected to frequent pruning practices a big amount of biomass (branches and leaves) results (one-five tons per cultivated hectare) (Tesfaye *et al.,* 2022). Otherwise, it is well known that the avocado leaves are rich in bioactive compounds, especially polyphenols, and in fact, the indigenous populations of central America used them to prepare teas or infusions against different diseases, such as neurodegenerative and cardiovascular diseases, cancer, etc. (Jimenez *et al.,* 2021).

Table 1 Amount* of bioactive compounds in avocado fruits and leaves.

Class of substances	Pulps	Seeds	Peels	Leaves
Total phenolic content (mg GAE/100g DW)	60-535	7.73-38.98	246-535	1.70 – 2.40
Total flavonoid content (mg QE/100g DW)	49.5-396	47.9	44.3-243	552
Total carotenoids content (mg/100g DW)	0.3-1.2	0.07-0.9	0.89-2.6	0.3
Antioxidant activity (DPPH µM TE/g DW)	0.8-8.3	128-240	39-189	57.8-110

* (Jimenez *et al.,* 2021); GAE: gallic acid equivalent; QE: quercetin equivalent; DW: dry weight.

Due to the climatic changes, avocado production is largely increasing in the Mediterranean area with the Hass variety the most appreciated and cultivated in Sicily for its intense taste and creamy pulp (Nyakang'i *et al.,* 2023); even if other varieties are cultivated, too such as Bacon, Fuerte, and Reed; the varieties distinguish each other not only for the sensory features but also for the amount of bioactive compounds. As regards the avocado varieties cultivated in Sicily, to the best of our knowledge, no information is reported in the literature on the fruit composition. Here, as happens in the countries of origin, the large quantity of waste and by-products represents one of the main problems of avocado production (Salazar-Lopez *et al.,* 2020; Araújo *et al.,* 2018). Considering the composition and the amount of bioactive compounds in leaves, peels, and seeds (Jimenez *et al.,* 2021), they are a promising material to produce innovative ingredients for functional foods; in the context of a circular economy, converting them into value-added goods could be a solution to improve the economic and environmental

sustainability of the avocado production.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) **Characterization of avocado by-products and wastes** through proximate composition, including total dietary fiber (A1.1) and bioactive compounds (A1.2).

A2) **Production of flours.** Defatted pulps, seeds, peels, and leaves will be dried through different drying green technologies and milled to obtain flours.

A3) **Chemical, technological, and sensory characterization of the flours** through physical and chemical analyses (A3.1), technological (water absorption solubility, hydrophobicity, emulsifying and foam, water and oil absorption capacity, viscosity, and gelatinization) (A3.2) and sensory analysis (color and texture profile), and consumer acceptability (hedonic test, CATA test, survival analysis, preference maps) (A3.3).

A4) **Nutritional evaluation and probiotic activity of the flours** through protein digestibility, glycemic index, and microbial survival studies.

A5) **Application of the flours** in baked goods and/or meat products.

A6) **Writing and Editing** of the PhD thesis, scientific papers, and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Characterization of avocado by-products and waste***																								
	1)Proximate composition																								
	2)Bioactive compounds																								
A2)	***Production of flours***																								
A3)	***Chemical, technological, and sensory characterization of the flours***																								
	1)Physical and chemical analyses																								
	2)Technological analyses																								
	3)Sensory analysis																								
A4)	***Nutritional evaluation and probiotic activity of the flours***																								
A5)	***Application of flours***																								
A6)	***Thesis and Paper Preparation***																								

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Evaluation, development and implementation of a mobile application as an educational and empowerment tool to promote healthy and sustainable diets in university students

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This PhD thesis research project aims to promote healthy and sustainable diets and reduce food waste in university students through the realization of a mobile application that detects consumption in university canteens in Parma with a Computer Vision system and provides tailored nutritional recommendations that also aims to improve food literacy and food-neophobia. The PhD is founded by ON Foods - Research and innovation network on food and nutrition Sustainability, Safety and Security – Working ON Foods[1]

Valutazione, sviluppo e implementazione di una applicazione mobile come strumento educativo e di empowerment per promuovere diete sane e sostenibili negli studenti universitari

Questo progetto di tesi di dottorato mira alla promozione di diete sane e sostenibili e alla riduzione di sprechi alimentari in studenti universitari attraverso la realizzazione di una applicazione mobile che rilevi i consumi nelle mense universitarie con un sistema di Computer Vision e fornisca indicazioni nutrizionali personalizzate atte anche a migliorare la food-literacy e la food-neophobia.

1. State-of-the-Art

Worldwide overweight and obesity has nearly tripled since 1975 (WHO, 2021). At least 2.8 million people die each year as a result of being overweight or obese, and an estimated 35.8 million (2.3%) of global DALYs are caused by overweight or obesity (Dai *et al.*, 2020). Public health systems are under increasing pressure from non-communicable diseases (NCDs) including obesity, diabetes, heart disease and some cancers. At the same time, food systems are a substantial contributor to climate change, biodiversity loss and the depletion of natural resources. Changes in food systems are needed not only to address the rise in diet-related NCDs, but to promote a shift towards an environmentally sustainable future.
Mediterranean diet (MD) is a milestone to aim for by reducing mortality in the general population and is also associated with low environmental impact (Tilman and Clark, 2014). In last decades adherence to MD decreased in Mediterranean countries, resulting in a rise of western dietary pattern (associated with a greater environmental impact and a higher risk of NCDs). To reverse this trend, innovative educational models are needed to promote healthy and sustainable diets in specific populations. Supportive environments and communities are fundamental in shaping people's choices, by making healthier foods and regular physical activity the easiest choice (the most accessible, available and affordable), and therefore preventing overweight and obesity. Moreover, to influence food choices, there is a need to identify and understand determinants of people's behavior focusing both on the interpersonal level and the food environment, which refers to the physical, economic, socio-cultural and policy conditions that shape access, affordability, safety, and food preferences and includes the social network, physical context, and policy-related factors (*The Factors That Influence Our Food Choices*). People more conscious of current environmental issues are more prone to shift toward more sustainable eating habits. Therefore, increasing peoples' literacy about health and environmental impact of food systems seems to be a promising strategy for raising awareness and building the capacity to adopt food practices that enhance health and well-being.
A mobile application called NUBI (NUtrizione BImbi) has been developed and tested in Parma (Rosi *et al.*, 2016). Parents of children attending primary schools in Parma were given nutritional advice and suggestions for dinners and weekends in relation to what their children ate at school. Currently in literature there are not many validated models that uses new technologies recognized as effective in changing eating habits. Nevertheless, their main potential to enhance dietary assessment is through more cost- and time-effective, less laborious ways of data collection and higher subject acceptance (Illner *et al.*, 2012). Recently, Computer Vision for food recognition (by using Convolutional Neural Networks) have had a big step forward, achieving about 79% of food and tray

[1] Project funded under the National Recovery and Resilience Plan (NRRP) Mission 4 Component 2 Investment 1.3 - Call for proposals No. 341 of 15 March 2022 of Italian Ministry of University and Research funded by the European Union – NextGenerationEU; Project code PE00000003, Concession Decree No. 1550 of 11 October 2022 adopted by the Italian Ministry of University and Research, CUP D93C22000890001

recognition accuracy (Ciocca, Napoletano and Schettini, 2017). Computer vision could be implemented in university canteens in Parma to assess food consumption and based on it, provide customised nutritional and environmental recommendations to students. The use of a mobile application as a communication tool to convey educational content in terms of nutrition and sustainability, together with the improvement of the food offer and the implementation of a supportive environment, could make university canteens a strategic setting to advocate healthy and sustainable nutrition in both university staff and students (Krattenmacher *et al.*, 2023).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Literature review of educational models (A1.1) aimed to drive people to healthy and sustainable diets.

A2) Food consumption habits, food choices and plate waste (A2.1), nutrition and food sustainability knowledge (A2.2) of Parma University students attending the University cafeteria will be investigated to obtain a baseline reference.

A3) The educational material, the tools, and information on the food offered in the canteen will be made available to the students in a digital application (A3.2) the customization of which could be addressed thanks to the support of UNIMIB (A3.1).

A4) After discussion about feasibility with the caterer, optimized menus (A4.1) will be developed with the use of more sustainable recipes, together with the dining rooms re-designed (A4.2) to promote users' engagement and increase students' literacy.

A5) During and after the intervention the same outcome variables food consumption habits, food choices and plate waste (A5.1), nutrition and food sustainability knowledge (A5.2) will be monitored to assess the efficacy of the intervention itself.

A6) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
A1)	***Educational models***																																
	1) Literature review																																
A2)	***Baseline assessment***																																
	1) Consumption and waste																																
	2) Sustainability habits																																
A3)	***Mobile application***																																
	1) App customization																																
	2) Educational material																																
A4)	***Environment optimization***																																
	1) Optimization of menu																																
	2) Dining room design																																
A5)	***Intervention assessment***																																
	1) Consumption and waste																																
	2) Sustainability habits																																
A6)	***Thesis and Paper***																																

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Genomic characterization of Lactic Acid Bacteria strains for novel pro- and post-biotics development

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This PhD thesis research project is aimed to exploit the autochthonous microbial communities of typical Italian fermented products to design new microbial consortia that have pro- and postbiotic properties. A plant-based psychobiotic yogurt will be developed to deliver neurotransmitter-producing strains and/or their psychobiotic metabolites into the gut and tested in a gut ecosystem simulator (SHIME), monitoring changes in the microbiome and metabolome. The expected results will open new avenues towards modulating the microbiome for mental health, linking health promotion with a sustainable food product.

Caratterizzazione genomica di ceppi di batteri lattici per lo sviluppo di nuovi pro e post-biotici

Questo progetto di tesi di dottorato ha lo scopo di sfruttare le comunità microbiche autoctone dei prodotti fermentati tipici italiani per progettare nuovi consorzi microbici che abbiano proprietà psicobiotiche. Uno yogurt psicobiotico a base vegetale sarà sviluppato per trasferire ceppi produttori di neurotrasmettitori all'intestino umano e testato in un simulatore dell'ecosistema intestinale (SHIME), monitorando i cambiamenti nel microbioma e nel metaboloma. I risultati attesi apriranno nuove strade verso la modulazione del microbioma per la salute mentale, collegando la promozione della salute all'utilizzo di un prodotto sostenibile.

1. State-of-the-Art

Improving human health through modulation of microbial interactions during all phases of life is an evolving concept that is increasingly important for consumers, food manufacturers, health-care professionals and regulators. Postbiotics is a research area of great relevance within the field of functional foods. The term 'postbiotics' is increasingly found in the scientific literature, and it means a "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (Salminen *et al*., 2021). Recent studies suggest the viability of bacteria may not be necessary to achieve health-promoting effects with undoubted advantage to circumvent the problem of acquisition of antibiotic resistance genes and virulence factors. The use of postbiotics for human health is still at a preliminary stage. The growing volume of genomic information may facilitate systematic efforts to determine the metabolic pathways that may lead to obtain the desired postbiotic metabolites. Of notable interest in the field of postbiotics is the production of molecules with a beneficial effect on the nervous system. Lactic acid bacteria (LAB) have been reported to produce neuroactive molecules, capable of modulating mood and cognition in humans (psychobiotics). Fermented foods (FFs) may be considered as a still underexplored reservoir of microbial resources of beneficial microbes, that may positively affect human mental health, releasing molecules with the potential to modulate pathways of the microbiome–gut–brain axis. Indeed, it was recently suggested that a diet rich in fermented food may positively impact on stress (Berding *et al*., 2023). Therefore, In Table 1, the main neuroactive molecules produced by bacteria within the human gut are reported, including their precursors, and their regulatory functions (Casertano *et al*., 2022).

Table 1 Representative list of neurotransmitters produced from bacteria within the human gut, precursors, and their regulatory functions.

Neuroactive compound	Precursors	Genus	Regulatory function
Gamma-aminobutyric acid (GABA)	Glutamate	*Lactobacillus, Bifidobacterium, Bacteroides*	Stress responsiveness, anxiety
Acetylcholine	Choline	*Lactobacillus, Bacillus*	Encoding of new memories
Dopamine	Tyrosine	*Lactococcus, Lactobacillus, Streptococcus*	Motivational decision-making
Serotonin	Tryptophan	*Lactococcus, Lactobacillus, Streptococcus*	Modulation of intestinal secretion and motility; Brain development

Based on this background, the thesis project aims to exploit the autochthonous microbial communities of typical

Italian fermented foods to design new microbial consortia capable of modulating the gut-brain axis through the direct production of neuroactive molecules during fermentation and/or through the modulation of intestinal microbiome activity in simulated digestion. A plant-based postbiotic yoghurt will be developed to deliver neurotransmitter-producing strains into the gut and tested in a gut ecosystem simulator, monitoring changes in the microbiome and metabolome. The expected results will open new avenues towards modulating the microbiome for mental health, linking health promotion with sustainability benefits.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) **Developing a collection of Lactic Acid Bacteria from typical Italian FFs:** LAB will be isolated and selected from traditional Italian FFs, including cheeses, sourdoughs used for the manufacturing of traditional breads and fermented olives (A1.1); Metagenome of FFs will be screened for the presence of microbial genes/pathways related to the production of neurotransmitters and neuroactive molecules (A1.2);

A2) **Developing three microbial consortia with postbiotic activities:** Strain testing for postbiotic and pro-technological activities in vitro. The production of neurotransmitters will be tested using broth containing monosodium glutamate, tyrosine, choline or tryptophan, precursors for GABA, catecholamine, acetylcholine and serotonin production. Supernatants will be analysed by LC-MS/MS. Strains will be also tested for pro-technological activities, considering their ability to ferment different plant-based matrices (rice, legumes, cereals) (A2.1); Strains showing the best pro-technological performances and being able to release significant concentration of neuroactive molecules during fermentation will be considered suitable for fermentation of a yogurt-like (YL) product and so used to prepare the microbial consortia (A2.2)

A3) **Developing a plant-based, yogurt-like product with psychobiotic properties:** The microbial consortia will be tested for their ability to ferment different plant-based matrices like gelatinized mixture of rice and non-conventional flours (e.g., from legumes, pseudo- or minor-cereals) (A3.1); their growth and acidification ability will be monitored, as well as the ability to release neurotransmitters in the matrices (A3.2);

A4) **Testing the product in the SHIME model:** the best performing YL product will be tested in m-SHIME. During each run, a daily consumption of one portion (125g) of the YL product for 2 weeks will be tested (A4.1); all samples will be analyzed before microbiome stabilization, after stabilization, at different time-points during fermentation and at the end of the treatment. Microbiome will be analysed by shotgun metagenomics and neurotransmitters will be detected in the different SHIME compartments (A4.2);

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity_____Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Psychobiotic activities in Italian FFs***																								
	1) FFs collection and strain isolation																								
	2) Metagenomic characterization																								
A2)	***Developing microbial consortia***																								
	1) Strain testing																								
	2) Consortia development																								
A3)	***Developing a YL product***																								
	1) Formulation																								
	2) Monitoring of acidification ability and psychobiotic properties																								
A4)	***Testing in the SHIME model***																								
	1) SHIME model tuning																								
	2) Processing of collected data																								
A5)	***Thesis and Papers Preparation***																								

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Analysis of the socio-economic performances of producers' associations and their propensity to introduce innovations in the agri-food sector

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This PhD thesis research aims to examine the innovation processes in the agricultural sector, deepening the propensity to introduce innovation by producers' associations such agricultural cooperatives in Apulia region (southern Italy). This research will contribute to: (i) enhance knowledge about the factors that favour the innovation adoption of agri-food cooperatives, (ii) verify the effectiveness of the applied methodology and the achievement of interventions objectives and, (iii) propose corrective and/or improvement actions.

Analisi dei risultati socioeconomici delle imprese in forma associativa e della loro propensione all'introduzione di innovazioni nell'agroalimentare

Questo progetto di tesi di dottorato si pone come obiettivo la disamina dei processi di innovazione nel settore agricolo, con un approfondimento sulla propensione ad introdurre innovazione da parte delle imprese associate quali le cooperative agricole della regione Puglia (Italia meridionale). Questa ricerca contribuirà a: (i) ampliare le conoscenze in merito ai fattori che favoriscono l'implementazione di innovazione da parte delle imprese cooperative agroalimentari, (ii) verificare l'efficacia della metodologia applicata ed il raggiungimento degli obiettivi di intervento e, (iii) proporre azioni correttive e/o di miglioramento.

1. State-of-the-Art

Europe 2020 Strategy proposes a new model of growth and economic development - smarter, more sustainable and more inclusive - calling for research and innovation to operate in a strong connection with the needs of people and businesses. Consistently, the 2014-2020 programming for the use of European Funds and related national co-financing has provided, among its thematic objectives, to strengthen research, technological development and innovation and promote the competitiveness of small and medium-sized enterprises (SME), the agricultural sector and the fisheries and aquaculture sector in a logic of sustainable growth (Piano strategico per l'innovazione e la ricerca nel settore agricolo alimentare e forestale 2014-2020). Innovation represents a key factor in addressing the challenges of the future concerning environmental sustainability and increasing business competitiveness. In this context, cooperation could be an incentive factor for innovation in the agri-food sector, contributing to the balancing of market relations according to bargaining power and distribution of added value. The relationship between demand and supply of innovation is often impeded by difficult communication and physical limits between those who propose innovation and those who must adopt it. Therefore, it is necessary to facilitate the relationship between researchers and companies. The research project will concentrate on enhancing the propensity for innovation of individual companies and production cooperatives in Apulia's agricultural sector. Consequently, this analysis will help to improve the efficiency and effectiveness of the activities conducted by the associated agri-food companies and it will favour the positive and concrete development of local communities. To achieve the stated objectives, a theoretical context analysis has been undertaken, as a first step of this doctoral research, through the bibliographic research's activity aimed at carrying out a systematic literature review. The main objective is to identify methodologies, theories or tools to measure the innovation impact in the agri-food sector and, secondly, to subdivide the results obtained according to specific classification criteria, that could bring out impact categories worthy of further study. More specifically, the aim is to focus attention on the factors that favour the innovation adoption by agri-food companies and on the effects that innovation can have on economic, social and environmental sustainability.
Therefore, using the *focus group* technique have been selected keywords used in the search strings: "Innovation"; "Agri-food"; "Assessment"; "Indicators" and "Agriculture", and have been identified the following six research questions:

1. DEFINITION OF INNOVATION;
2. TEORY OF INNOVATION;
3. DETERMINANTS OF INNOVATION;
4. BENEFITS OF INNOVATIVE AGRICULTURE ADOPTION;
5. TYPES OF INNOVATION TECHNOLOGY IN FARMER LEVEL;
6. TYPES OF ASSESMENT METHODS OF AGRICULTURE INNOVATION.

The Scopus database was used for material collection how it was made by Silvestri *et al.* (2022). This paper has been chosen as a model for the methodology used. Scholars consider Scopus to be among the best databases to

produce a reliable bibliometric survey (Durán-Sánchez *et al.*, 2018). Scopus offers a high level of singularity (Sánchez *et al.*, 2017) and broad data coverage (Salim *et al.*, 2019), making it one of the most comprehensive and comprehensive scientific databases (Chadegani *et al.*, 2017). In methodological terms, a literature review allows investigation of a given topic through both qualitative and quantitative content analysis (Hill, 1995; Seuring and Muller, 2008). The search strings used in Scopus have been "Agri-food" OR "Agriculture" AND "Innovation" AND "Assessment" OR "Measurement" OR "Evaluation". In the Scopus search, the research criteria were "Title, Keywords, Abstract". Finally, the following filters have been set:

- Year (2015 to 2023);
- Country (Italy);
- Publication stage (Final);
- Language (English);
- Subject area (Environmental Sciences; Agricultural and Biological Sciences; Social Sciences; Economics, Econometrics and Finance; Business, Management and Accounting);
- Document type (articles and reviews).

The search on Scopus produced 1185 results. Of these, 113 were selected, depending on their ability to answer research questions. These articles and reviews will be the basis of the proposed activities.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Innovation adoption** determination of the factors affecting the adoption of innovation (A1.1) and benefits of innovative agriculture adoption (A1.2) to focus on the factors that favour the adoption of innovation of agri-food companies and the benefits of innovation adoption.

A2) **Assessment method** determination of the types of assessment methods (A2.1) and indicators (A2.2) used by the selected articles to identify the mathematical model capable of measuring innovation. More specifically focusing on specific analysis models that will analyse economic, social, and environmental sustainability.

A3) **Method and data collection** to define the method to be used (A3.1) and to perform data collection (A3.2). Once the method has been defined, it is necessary to prepare the dataset of the associated agri-food companies to be examined. It will be necessary to choose supply value and production sector.

A4) **Application of the method to the case study** the dataset developed in the previous activity.

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project during the next 2 years (2023-2025).

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Innovation adoption***																								
	1) Factors affecting																								
	2) Benefits																								
A2)	***Assessment method***																								
	1) Assessment methods																								
	2) Indicators																								
A3)	***Method and data collection***																								
	1) Method																								
	2) Data collection																								
A4)	***Application***																								
A5)	***Thesis and Paper Preparation***																								

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Salim N, Ab Rahman MN, Abd Wahab D (2019) A systematic literature review of internal capabilities for enhancing eco-innovation performance of manufacturing firms. *J Clean Prod* **209**: 1445–1460.

Seuring S, Muller M (2008) From a literature review to a conceptual framework for sustainable supply chain management. *J Clean Prod* **16**: 1699–1710.

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Pilot and industrial scale design, optimization and development of innovative plant-based functional food and supplements

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This PhD thesis research project is aimed at developing biotechnological protocols for obtaining new functional foods and beverages. The research will involve the set-up of fermentation protocols, including the selection of *i*) ingredients, *ii*) starters, and *iii*) process parameters. The optimized formulation will undergo a more in-depth characterization of the compounds responsible for the functional activities as well as their effect on human health, followed by the industrial scale-up of the experimental prototypes.

Strategia sperimentale per la progettazione ottimale di alimenti e bevande funzionali

Questo progetto di dottorato è finalizzato alla messa a punto di protocolli biotecnologici per lo sviluppo di alimenti e bevande funzionali, attraverso l'uso di batteri lattici selezionati e la selezione di ingredienti vegetali. La formulazione ottimizzata subirà una caratterizzazione più approfondita dei composti responsabili delle attività funzionali e del loro effetto sulla salute umana, seguita dallo scale-up industriale dei prototipi sperimentali.

1. State-of-the-art

The concerns about environmental impact and sustainability of animal-based diets, as well as human health issues thereof related, have fuelled consumer demand for dairy alternatives, paving the way to plant-based yogurt-like (YL). The term YL refers to products similar to conventional yogurt in terms of structure, sensory properties and ability to keep lactic acid bacteria alive and viable for a long time, however, obtained from matrices other than milk. Cereals, pseudocereals and legumes are widely used as main ingredients in YL formulations given the wide availability and moderate cost and since they still represent the main source of macro and micronutrients worldwide. These grains represent alternative protein sources to animal-derived ingredients being rich in proteins of high biological value, fibres, and bioactive compounds (Gobbetti et al., 2020). Nevertheless, the nutritional and functional value of plant matrices can be compromised by the presence of anti-nutritional factors (ANF), which can adversely affect their nutritional and sensory profile. The most common ANF in plants are condensed tannins, saponins, phytic acid, α-galactosides and trypsin inhibitors. Fermentation, in addition to having a positive impact on the nutritional value, sensory and technological properties of vegetables products, has been thoroughly investigated as a biotechnological process capable of reducing the impact of ANF (Gobbetti et al., 2020).
Being characterized by proteins of different nature compared to milk once, plant protein rarely precipitate due to acidification and this is one of the major problems related to the production of YL from vegetable matrices. YL are mainly obtained after the fermentation of aqueous extracts or suspensions in water of cereal flours, pseudocereals, legumes, homogenized fruits and over the years there have been different attempts to obtain a structure similar to that of traditional yogurt. Moreover, often YL production process is long and expensive, because compared to animal sources plant matrices have lower protein content with different coagulation modes thus requiring structuring agents and emulsifiers. The acidification caused by fermentation also leads to a destabilization of plant protein causing, during storage, a loss of viscosity with consequent separation of the aqueous phase (Bernat et al., 2014), hence, different solutions can be adopted. Although not responding to the growing demand for clean-label products, additives can be used. Alternatively, lactic acid bacteria strains able to produce exopolysaccharides (EPS) which improve YL structural properties can be employed as starters or starch gelatinization can be carried out before fermentation. Starch gelatinization can also prevent phase separation while reducing contamination by endogenous microorganisms before inoculation of selected starters (Pontonio et al., 2020). Based on the above considerations the aim of this research project is to set-up biotechnological protocols for the development of functional YL which comply with the concept of clean label.

2. PhD Thesis Objectives and Milestones

This PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Exploiting the possibilities of functionalization (enrichment in bioactive compounds) of a rice-based matrix** intended as main base for YL production, will be achieved through the following operational phases:

Screening of i) different plant-based ingredients to be used for the YL fortification (A1.1); ii) lactic acid bacteria selected for pro-technological and functional characteristics (e.g. GABA and EPS synthesis) (A1.2). The analytical plan for the full set of the YL prototypes will include the determination of the nutritional label, kinetics of acidification, total titratable acidity; concentration of organic acids (lactic and acetic acids), free amino acids (including the functional GABA), and peptides. Microbiological analysis will also be performed during the different bioprocessing stages and during storage in refrigerated shelf-life. Aiming at defining the technological properties and EPS synthesis by the starter, viscosity will be also determined. The recipes will be then optimized, based on the results obtained from the nutritional and functional characterization of the YL (A1.3).

A2) **The experimental prototypes will be evaluated through a sensory characterization** of the product (A3.1) and the most interesting theses, will be object of industrial scale-up of the production process (this activity will be carried out during the 6-months periods in Celery company (A2.2).

A3) **A nutritional-functional characterization of the selected YL prototypes** will also include the evaluation of antioxidant activity by *in vitro* and *ex-vivo* (on human or animal cell cultures) assays; the characterization of the molecules responsible for the antioxidant activity (this activity will be performed during the 6-months period at University of Granada, Spain); the evaluation of the *in vitro*-protein digestibility and *in vitro* glycaemic index (A3.2).

A4) **Evaluation of YL effects on microbiota.** Once the development of the selected prototypes has been completed, collaborative investigations will be carried out aiming at determining the effect of the new YL products on the gut microbiota and on the diet (A4).

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1: *Gantt diagram for this PhD thesis project.*

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Possibilities of functionalization***																								
	1) Screening of ingredients																								
	2) Screening of LAB																								
	3) YL optimization																								
A2)	***Sensory characterization and scale-up***																								
	1) Sensory characterization																								
	2) Industrial scale-up																								
A3)	***Nutritional-functional characterization***																								
	1) Evaluation of antioxidant activity																								
	2) Evaluation of protein digestibility and glycaemic index																								
A4)	***Evaluation of YL effects on microbiota***																								
A5)	***Writing and editing***																								

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Digital innovation for sustainable intensification in cereals sector. An economic valuation of contractual agreements and CAP eco-schemes adoption in bakery value chain in Italy

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Tutor: Prof. Emanuele Blasi

This PhD thesis research project aims to implement current information and studies on the actual economic benefits of adopting sustainable agricultural practices in a raw materials supply chain. It also intends to evaluate the willingness to adopt digital tools related to support contractual relationships and to monitor raw material and values flows among bakery supply chain actors. Finally, the project aims to update the analysis of synergy between private initiatives and public policies for sustainable agricultural schemes.

Innovazione digitale per l'intensificazione sostenibile nel settore cerealicolo. Una valutazione economica degli accordi contrattuali e dell'adozione degli eco-schemi della PAC nella filiera dei prodotti da forno in Italia

Il progetto di tesi di dottorato mira a implementare le informazioni e gli studi attuali sugli effettivi benefici economici derivanti dall'adozione di pratiche agricole sostenibili nella filiera di approvvigionamento delle materie prime. Si intende inoltre valutare la disponibilità ad adottare strumenti digitali relativi al supporto dei rapporti contrattuali e al monitoraggio dei flussi di materie prime e valore tra gli attori della filiera dei prodotti da forno. Infine, il progetto mira ad aggiornare l'analisi delle sinergie che si potrebbero instaurare tra iniziative private e politiche pubbliche per i sistemi agricoli sostenibili.

1. State-of-the-Art

The dominant paradigm in European agriculture is characterized by highly specialised and industrialized production. It is based on intensive use of agrochemicals, monocultures and few crops and varieties, and contributes to greenhouse gas emissions, water pollution, soil degradation and loss of biodiversity (Kleijn et al., 2019). The role of farmers in mitigating impacts on natural resources is becoming increasingly central to the decision-making process of policies such as the CAP. Among the solutions proposed by CAP, is the renewed intention to increase arable and crops diversification and to protect biodiversity by farmer and agricultural system (Bonke e Musshoff,2020).

The new CAP 2023-2027 introduces among the good agricultural and environmental conditions (GAEC) crop rotation in arable lands (GAEC7) and a minimum percentage of arable land to be allocated to area or non-productive elements (GAEC8). To this are added the new eco-schemes, the main innovation in the green architecture of the CAP. As mandatory instruments, they would oblige Member States to allocate a proportion of their Pillar 1 payments to schemes that would directly benefit the environment and climate.

The strategic plan of the CAP of Italy identifies 5 eco-schemes, in particular the eco-scheme 4 "extensive forage systems with rotation" that provides support for arable land in rotation of crops legumes and forage, and the eco-scheme 5 "Specific measures for pollinators" which aims to contribute to the preservation of biodiversity through the spread crops of bee interest and a sustainable and reduced use of pesticides (Strategic Plan for the CAP, Italy, 2021). Crop diversification, rotation patterns and flower strips are recently recognised as keys CAP measure to drive Italian agricultural sector towards sustainable path (Bonke e Musshoff 2020).

Numerous studies confirm the actual short- and long-term environmental benefits that can be derived from the adoption of sustainable farming practices, and therefore changes of this type are considered win-win strategies from the environmental point of view, however, the literature reports a smaller number of studies that investigate the potential income from the adoption of sustainable production methods and a large part of these studies refer to a small scale, often family-run business and located in developing contexts (Schleich et al., 2019).

Despite their benefits, implementing such practices often implies (initial) increased costs for farmers (Schleich et al., 2019) and a change in practices often requires the reorganization of relations with downstream partners at value chain level (Meynard et al., 2017).

Extant literature, in fact, mostly discusses forms of value chain organisation, including contract farming that have enabled farmers to adopt new practices aiming to improve product quality, or comply with food safety standards (Kumar et al., 2018). Often contractual arrangements are needed to provide premium prices to farmers to incentivise the adoption of practices and the implementation of quality standards (Banterle and Kuijpers, 2019). Nonetheless, studies suggest that there is no blueprint contract to encourage adoption (Meynard et al., 2017) and

designing adequate contracts and value chain configurations to support the adoption of sustainable practices can be a complex process (Pancino et al., 2019). The transition towards sustainability commits the agri-food chains to define new business models that encourage the adoption of new agro-ecological practices by farms without undermining the volumes of the supplies. Incentives such as linking subsidies to the adoption of sustainable practices, particularly through the CAP, may be a mechanism to enhance participation, and reach additional farmers (Weituschat et al., 2023). According to this paradigm, the question arises whether the new CAP aid scheme encourages the participation of specialised agricultural enterprises in private sector schemes.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) **Analysis of the context**: literature review on sustainable cultivation practices, on the adoption of digital innovations for sustainable intensification (A1.1) and definition of case study (A1.2).

A2) **Farms data collection:** structural and economic data will be collected to describe farms involved in CAP and in private scheme (A 2.1) Stable panel identification (A 2.2). Provide tools to assess farms costs and benefits in different practices adoption scenario (A2.3).

A3) **Study and identification of tools and methods**: attend advance courses on socioeconomics analysis and econometrics techniques (A3.1). Development and test a useful methodology to address and validate the research questions (A 3.2).

A4) **Development of analysis:** adoption scenarios characterization (A 4.1). Impact assessment of public polices and business model adoption at farm and value chain level (A 4.2). Outcome valorisation by stakeholder consultation (policy makers and business managers of the value chain)

A5) **Writing and Editing:** Phd thesis, scientific papers, and oral and/or poster communications will carry on during PhD period.

Table 2 Gantt diagram for this PhD thesis project.

Activity	Trimesters	3	6	9	12	15	18	21	24	27	30	33	36
A1)	***Context analysis***												
	1) Literature review												
	2) Identification of case study												
A2)	***Data collection***												
	1) Farms data collection												
	2) Stable panel identification												
	3) Provide tools useful to assess farms costs and benefits												
A3)	***Methodology***												
	1) Advance courses on econometrics techniques												
	2) Development and test of the methodology												
A4)	***Development of analysis***												
	1) Adoption scenarios characterization												
	2) Impact assessment of public policies and private initiatives												
	3) Results analysis and valorisation												
A5)	***Thesis and Paper Preparation***												

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Protein and polyphenols interactions: functionality in food and biological systems

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This PhD research project aims at exploring the interactions between proteins/peptides and polyphenols. Food and beverage systems will be used as experimental model to explore the impact of the interactions on physicochemical characteristics of both proteins/peptides and polyphenols and on food quality. Moreover, the physiological effect of consuming the protein-polyphenol complexes will be explored by *in vitro* model systems and *in vivo* studies focusing on specific metabolites and markers of function of biological systems.

Interazioni tra proteine e polifenoli: funzionalità negli alimenti e sistemi biologici

Questo progetto di ricerca di dottorato mira a esplorare le interazioni tra proteine/peptidi e polifenoli. Sistemi di alimenti e bevande saranno utilizzati come modello sperimentale per esplorare l'impatto delle interazioni sulle caratteristiche fisico-chimiche di proteine/peptidi e polifenoli e sulla qualità degli alimenti. Inoltre, l'effetto fisiologico del consumo dei complessi proteine-polifenoli sarà valutato attraverso sistemi modello in vitro e studi *in vivo* incentrati su metaboliti specifici e marcatori di funzione dei sistemi biologici.

1. State-of-the-Art

Dietary proteins are important food components providing beyond nutritional properties, both technological (solubility, foaming and emulsifying) and bioactive properties due to peptides can be originated over digestion. Polyphenols are a class of phytochemicals, recognized for mitigation of several chronic and degenerative diseases including obesity, cardiovascular disease, and neurodegeneration (Foegeding *et al*., 2017). These two nutrients co-exist in many foods and can easily form aggregates because of processing and after consumption when being co-ingested and interact in the gastrointestinal tract (GIT) (Zhang *et al*., 2021). The resulting interactions are classified into covalent interactions which are irreversible and stable and non-covalent interactions which are driven by hydrogen bonds, Van Der-Waals forces, hydrophobic and electrostatic interactions and are reversible. The generation as well as the strength of these interactions is modulated by intrinsic factors, depending on the type, the amount and the ratio of both molecules involved, and extrinsic variables such as pH, temperature, and the influence of other constituents. The effects of these interactions are still unclear due to inconsistent evidence. Such intermolecular associations induced changes in protein conformational structures leading up to modulate their solubility and other technological properties which are essential requirements for protein food applications. Moreover, the antioxidant activity of polyphenols can be masked by proteins due to the blockage of their reactive groups by intramolecular interactions (Ozdal *et al*., 2013). The changes caused by these complexes, result in different digestive behaviors of protein and polyphenols compared to parental free molecules. Protein digestion can be reduced by the formation of indigestible aggregates or by the possible inactivation of digestive enzymes (Zhang *et al.,* 2021). However, the presence of phenolics could also induce partial unfolding of protein structures, therefore increasing accessibility of the susceptible peptide bonds and digestibility (Jiang *et al*., 2018). Likewise, the bioaccessibility and the metabolic fate of complexed polyphenols is challenged by many factors. Polyphenols need to be released from the food matrices into the gastrointestinal tract and be absorbed, to exert their biological activities in peripheral organs (Ribas-Agustí *et al.,* 2018). Few in *vivo* and cell-based transportation studies reported either a deteriorative effect of proteins on the bioavailability of co-ingested polyphenols or an increased uptake due to an improvement in stability of bonded polyphenols which are protected by proteins from being degraded. Furthermore, during digestion, the interactions can be partially dissociated due to environment conditions and new associations might also occur between polyphenols and peptides leading to new potential bioactive complexes (Zhang *et al.,* 2021).

The objective of this PhD project is to study protein/peptide-polyphenol interactions to optimize functional properties in food and health-promoting effects in humans upon food consumption. To this purpose, food/beverage systems containing animal/plant proteins/peptides and polyphenols will be designed, developed and used to explore the interactions between proteins and polyphenols as well as the impact on food properties and protein/polyphenol bioaccessibilty, metabolism and functionality using both *in vitro* and *in vivo* approaches.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be divided into the following activities according to the Gantt diagram in Table 1:

A1) **Design, development and characterization of food/beverage systems** containing protein/peptide-polyphenol complexes (focus on pulse-based preparations and plant-based beverages also using by-products coming from agro-food industry).
A2) **Study of potential functionality** of the protein/peptide-polyphenol complexes in food/beverage systems by using *in vitro* studies (focus on protein and polyphenol digestibility, metabolism and nutritional/functional properties through bioaccessibility of aminoacids, bioactive peptides, digestive enzyme inhibitory activity, antioxidant activity, etc).
A3) **Evaluation of physiological effects** of consuming food/beverage containing protein/peptide-polyphenol complexes (focus on specific dietary metabolites and physiological markers) in human studies.
A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Design, development and characterization of food/beverage systems***																								
A2)	***Study of potential functionality of polyphenol-protein complexes in food/beverage systems in vitro***																								
A3)	***Evaluation of physiological effects in vivo***																								
A4)	***Thesis and Paper preparation***																								

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Study of persistence and characterization of the food-born zoonotic pathogen *Arcobacter* spp.

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This PhD project aims at delineating a sector-specific risk assessment related to the presence of *Arcobacter* spp. in poultry and slaughterhouse. Genes connected to antibiotic resistance and virulence will be determined through physiological tests and genome analysis. Gene regulation and response mechanisms under stress conditions of the bacterium will also be analysed. The results obtained will be useful in expanding knowledge of this microorganism, focusing on its pathogenic potential. Special attention will be paid to antibiotic resistance and the possible transmission of resistance factors to humans, considering its high significance for public health.

Studio della persistenza e caratterizzazione del patogeno zoonotico di origine alimentare *Arcobacter* spp.

Questo progetto di dottorato mira a delineare una valutazione del rischio specifico nel settore legato alla presenza di *Arcobacter* spp. nella filiera avicola e nei macelli. I geni legati all'antibiotico resistenza e alla virulenza verranno determinati tramite test fisiologici e analisi del genoma. Verrà inoltre analizzata la regolazione genica e i meccanismi di risposta in condizioni di stress del batterio. I risultati ottenuti saranno utili per ampliare le conoscenze su questo microrganismo, concentrandoci sulla sua potenzialità patogena. Verrà posta particolare attenzione all'antibiotico resistenza e alla possibile trasmissione dei fattori di resistenza all'uomo, considerando l'elevata rilevanza per la salute pubblica.

1. State-of-the-Art

Arcobacter spp. is a Gram-negative bacterium originally included in the *Campylobacteraceae* family and following a recent taxonomic revision, the genus was reclassified into the family *Arcobacteraceae* (On *et al.*, 2020). The species of greatest importance are *Arcobacter butzleri*, *Arcobacter cryaerophilus*, *Arcobacter skirrowii* and *Arcobacter thereius* as they are often associated to clinical conditions (Ramees *et al.*, 2017). They are responsible in humans for diseases such as bacteremia, endocarditis, peritonitis, gastroenteritis, and diarrhoea; numerous problems also occur in animals such as diarrhoea, mastitis, and abortions. The pathogenicity of this microorganism is still underestimated due to the lack of knowledge and misdiagnosis of infection, often attributed to *Campylobacter* spp. (Collado and Figueras, 2011; Ramees *et al.*, 2017).
The ingestion of contaminated food or water is considered the most likely route of transmission of these bacteria to humans (Ramees *et al.*, 2017). *Arcobacter* spp. has been isolated from the following food products: chicken meat, red meats (pork, beef and lamb), raw milk, seafood and vegetables (Müller *et al.*, 2020). Among these, poultry meat appears to have the higher percentage of samples in which *Arcobacter* spp. has been detected (Zacharow *et al.*, 2015). The distribution of *A. butzleri* throughout the food chain has been amply demonstrated through investigations on food products from the processing stage to retail and on ready-to-eat products. The unequivocal presence of this bacterium on the surfaces of food processing plants such as slaughterhouses or dairies is favoured by the ability to adhere to different materials and to form biofilm under different conditions. In addition, the adhesion of *A. butzleri* to food surfaces is also a source of cross-contamination (Ferreira *et al.*, 2019).
As for *Campylobacter* spp., closely related to *Arcobacter* spp., normally the infection caused by *Arcobacter* does not require antibiotic treatment. However, the severity or prolongation of symptoms may justify the use of antibiotics (Collado and Figueras, 2011; Ramees *et al.*, 2017). The emergence of antibiotic resistance phenomenon makes the use of antibiotics for clinical treatment less efficient (Collado and Figueras, 2011). Unlike *Campylobacter* antimicrobial susceptibility tests in *Arcobacter* species are not standardized. Many *A. butzleri* strains are resistant to clindamycin, azithromycin, ciprofloxacin, metronidazole, carbenicillin, and cefoperazone (Ramees *et al.*, 2017). Contamination of food by highly antibiotic-resistant bacteria is a public health issue considering the possibility of transmission to humans of genes linked to antibiotic resistance (Gungor *et al.*, 2023). Furthermore, despite the availability of numerous isolation techniques, there is no recommended standard method for isolation of *Arcobacter* spp.. Due to this limiting factors, many of the important cases may be undetected, resulting in underestimation of the prevalence and epidemiological status of this bacterium (Ramees *et al.*, 2017).

2. PhD Thesis Objectives and Milestones

Taking into consideration the aspects highlighted on *Arcobacter* spp., it is essential to expand the knowledge regarding its pathogenicity considering the high exposure to humans. With this purpose, *Arcobacter* spp. isolates from broiler carcasses (gut and neck skin) during slaughtering and from slaughterhouse surfaces will be analysed. This PhD thesis project can be divided into the following activities according to the Gantt diagram given in Table 1.

A1) **Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR** to identify the genetic diversity of the isolates.

A2) **Physiological tests of *Arcobacter* spp. isolates for virulence assessment:** tests for antibiotic resistance evaluation using the most used antibiotics in medical field; tests of cell colonization on mucus secreting human cell line (HT29-MTX-E12) and assessment of biofilm formation on microplate; detergent susceptibility analysis of isolates from slaughterhouse surfaces.

A3) **Whole Genome Sequencing:** following Illumina sequencing of *Arcobacter* spp. isolates, bioinformatic analyses will be carried out to annotate genes and obtain pangenome-related information (e.g., core and accessory genes).

A4) ***In vivo* pathogenicity studies**: evaluation of the pathogenic potential of selected *A. butzleri* strains in an *in vivo* murine model. Infection will occur by *gavage* method; the severity of the disease will be assessed for 15 days after infection by stool and blood analysis.

A5) **RNA sequencing analysis:** after the detergent treatment, the RNA sequencing analysis conducted on the samples will allow the highlighting of gene regulation and gene pathways involved in cellular metabolism and bacterial stress response mechanisms. The final goal is to assess the persistence of the pathogen under study.

A6) **Thesis and Paper preparation** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***ERIC-PCR***																								
A2)	***Physiological tests of Arcobacter spp. isolates***																								
	1) Antibiotic resistance tests																								
	2) Cell colonization and biofilm tests																								
	3) Detergent susceptibility tests																								
A3)	***Whole Genome Sequencing***																								
A4)	***In vivo tests***																								
A5)	***RNA sequencing analysis***																								
A6)	***Thesis and Paper Preparation***																								

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Microbial Biopolymers for Innovative Packaging to Increase Food Shelf-life and Safety

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The aim of this PhD research project is to develop the production of biopolymers for the formulation of food packaging from yeast cells or their metabolites, including those with antimicrobial activity, to extend the shelf life and safety of various food matrices. In particular, the biopolymers are produced from components of the cell walls of yeasts (glucans, mannans) or microbial metabolites such as pullulans and/or cellulose.

Biopolimeri microbici per imballaggi innovativi per aumentare la durata di conservazione e la sicurezza degli alimenti

Questo progetto di tesi di dottorato mira a mettere a punto la produzione di biopolimeri per la formulazione di un packaging alimentare a partire da cellule di lieviti o loro metaboliti, anche ad azione antimicrobica, al fine di incrementare la shelf-life e sicurezza di diverse matrici alimentari. In particolare, i biopolimeri saranno costituiti a partire da componenti della parte cellulare dei lieviti (glucani, mannani) o da metaboliti microbici quali pullulani e/o cellulosa.

1. State-of-the-Art

In recent decades, research and industry interest in food packaging has increased in the search for viable alternatives to the use of synthetic petroleum-derived films. Although petrochemical polymers such as polyethylene, polypropylene, polystyrene and polyamide are cost-effective products with good mechanical properties and excellent barrier properties to a variety of compounds such as oxygen, carbon dioxide, water vapor, and aromatic compounds, they have a significant negative impact on the environment as they are not biodegradable and come from non-renewable sources. A sustainable solution to reduce the problem of plastic accumulation in the environment and reduce the use of polymers from non-renewable sources could be the development of biodegradable polymers for environmentally friendly food packaging (Siracusa *et al.*, 2008). According to the literature, the natural polymers used to develop biodegradable materials are polysaccharides, proteins and lipids. Biopolymers are becoming increasingly important in food packaging as they can act as carriers for other molecules with antioxidant properties. In addition, thanks to their antimicrobial properties, active packaging can be produced to increase the shelf life and safety of various food products (Cerutti *et al.*, 2016). According to the literature, there are several materials that have the potential to be used as biopolymers, but they have not yet been fully explored. One such material is yeast biomass, whose cell walls are composed of glucans, mannoproteins and chitin. For example, the main polysaccharides that have been shown to be successful candidates for multicomponent film formation in combination with cell wall proteins are β-glucans, which make up about 55-65 % of the yeast cell wall. Using the yeast cell wall to form films that can be used as packaging also has the advantage of avoiding purification steps (Choque *et al.*, 2021). Currently, there are several methods to break down the yeast cell walls and separate the components from the intracellular components, such as sonication, but high-pressure homogenisation treatments may also be a sustainable non-thermal alternative. In addition to yeast cell walls, certain biopolymers such as pullulans produced by *Aureobasidium pullulans* or celluloses produced by various acetic bacteria could also be viable alternatives for obtaining biopolymers of microbial nature for use in packaging (Kraśniewska *et al.*, 2019). The production of yeast biomass and/or its metabolites from waste and by-products of the agri-food industry and their use as culture substrates for microbial growth could be another component of sustainability. Indeed, whey from the dairy industry, some by-products of the wine industry or vegetable and fish waste could be good growth substrates for potentially useful microorganisms due to their high organic matter content. Chitosan is undoubtedly one of the most researched polysaccharides. From 2016 to early 2021, more than 9000 articles have been published on the potential of chitosan in food packaging film formulation due to its non-antigenic, non-toxic, biodegradable, biocompatible, biofunctional and strong antimicrobial properties that can potentially extend the shelf life of food (Crini and Lichtfouse, 2019).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Bibliographic research** to identify the most suitable microorganisms for the recovery of cell wall constituents or their metabolites**.**

A2) **Screening of selected strains and optimisation of microbial performance** on agri-food industry wastes and by-products.

A3) **Selection of the most suitable methods and technologies** for breaking down cell walls and recovering the fraction of interest.

A4) **Characterisation of the obtained biopolymers** in terms of their antimicrobial and technological performance and film formulation.

A5) **Evaluation of the shelf life and safety of food matrices** in relation to selected packaging conditions.

A6) **Writing and Editing** of the PhD thesis, scientific papers, and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1)	***Bibliographic research***																		
	1) Identification of suitable microorganisms																		
A2)	***Screening of the selected strains and optimization of microbial performance***																		
	1)Selection and technological characterisation of selected yeast strains																		
	2) Optimisation of growth conditions for microorganisms on agri-food industry wastes and by-products																		
A3)	***Development of biotechnological processes for the recovery of biopolymers of interest***																		
	1) Application of microbial enzymes to obtain cellular compounds																		
	2) Application of high-pressure homogenisation, ultrasonication, pulsed electric fields																		
A4)	***Antimicrobial and technological characterisation of the obtained biopolymers and film formulation***																		
	1) Antimicrobial and antioxidant characterisation of biopolymers																		
	2) Film formulation and characterisation																		
A5)	***Evaluation of the shelf life and safety of food matrices in relation to selected packaging conditions***																		
A6)	***Thesis and Paper Preparation***																		

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Technological treatments to obtain high-quality food production

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This PhD thesis project aims to evaluate different technological treatments, useful for the reduction of the degradative phenomena that occur as a result of food processing. In particular, vegetable products will be subjected to chemical (e.g.natural antioxidants) and physical pretreatments (impregnation assisted by different systems) and then subjected to stabilization treatments. The efficacy of different operations will be evaluated through physicochemical, microbiological and antioxidant analysis in order to establish the best one to obtain a final product with excellent characteristics in terms of quality and shelf life.

Trattamenti tecnologici per ottenere una produzione alimentare di alta qualità

Questo progetto di tesi di dottorato ha lo scopo di valutare differenti trattamenti tecnologi, utili alla riduzione dei fenomeni degradativi che avvengono in seguito alla trasformazione degli alimenti. Nello specifico, alimenti di origine vegetale verranno sottoposti a pretrattamenti di natura chimica (es. antiossidanti naturali) e fisica (impregnazione coadiuvata da differenti sistemi) e successivamente sottoposti a trattamenti di stabilizzazione. L'efficacia dei diversi trattamenti verrà valutata attraverso analisi chimico-fisiche, microbiologiche ed antiossidanti al fine di stabilire il miglior trattamento che consenta di ottenere un prodotto finale con ottime caratteristiche dal punto di vista qualitativo e di durata di conservazione.

1. State-of-the-Art

The food processing and preservation concept has changed during the time, indeed, the initial aim was to obtain food products with a long shelf-life and innocuous for the human health, while today are required foods safety and characterized by a high content of nutrients and antioxidants. Particularly, the modern consumers are increasingly focused to purchase minimally processed fruits and vegetables, characterized by some aspects such as: health properties, convenience of use, high nutrition properties, extended maintenance of freshness. This has led to an intensification of research investment relatively to the development of alternative technique to extend the shelf life of this products and to maintain the nutritional properties.

One of the most important problems for this category of food products is represented by the more rapidly deteriorate than unprocessed raw materials, mainly because of damage caused by minimal processing methods. This implies that the shelf life of fresh cut fruits and vegetables has a decay and a series of typical symptoms, such as softening of the tissues, surface burnishing of the cut, decrease in nutritional value, presence of off taste and microbiological deterioration during storage (Ma *et al*., 2017). The application of technological treatments may be useful in overcoming this problem. Among the various emerging technologies used for treatments on food products in order to obtain safe products and with a higher quality we can mention the non-thermal treatments (NTT) the use of these treatments, in particular, has increased in recent decades (Morales-de la Peña *et al*., 2019); advantages over conventional heat treatments are as many as short processing times, greater process efficiency and better product quality, preventing colour, flavour and nutritional value alterations of vegetable products (Osae *et al*., 2020).

In addition, another very important aspect useful also to meet the needs of the consumer, is the use of natural preservatives, to replace the most critical synthetic additives that are used in the food system to extend food storage. These natural antioxidants can be obtained also by waste and/or by-products produced by agrifood companies. Wastes represent a resource of bioactive compounds such as polyphenols, essential oils, pigments, organic acids and functional additives, that after their recovery may be re-entered in the food system. These combined with technological treatments can greatly extend the shelf life of food and increase quality parameters. For example, with vacuum impregnation it is possible to obtain a product enriched from the nutritional point of view (Panayampadan *et al*., 2022). Different compounds (eg. nutritional and functional compounds, antimicrobial and antioxidant substances, organic acids and so on) can be introduced into the food and on the basis of type of compound chosen for impregnation, the final product will be characterized by better quality and greater shelf life (Tappi *et al*., 2016).

The following project will consider vegetable products in order to preserve and improve nutritional quality and extend shelf life. The addition of biomolecules inside food matrices, or their use for the realization of minimally processed fortified products, could bring improvements in the food industry, both as regards research through the extension of the shelf-life and to develop alternative techniques to minimise product quality losses after heat treatments.

Today is growing social attention to environmental issues that affect the excessive production of waste by agri-

food industries. Therefore, food enrichment with bioactive compounds recovered from by-products and the possible related increased shelf-life could reduce this environmental impact.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Application of pre-treatments:** evaluation of different pre-treatments (eg. vacuum impregnation with natural antioxidants, dipping in different solutions) (A1.1) application of pre-treatments on vegetable products and evaluation of physicochemical parameters (A1.2).

A2) **Stabilization/transformation treatments:** different types of cooking (eg. vacuum drying, semi drying, frying) (A2.1) evaluation of different conditions (time, temperature) and physicochemical parameters (A2.2).

A3) **Evaluation of shelf life**: evaluation of shelf life by physicochemical, microbiological analysis, sensorial and structural evaluation (A3.1).

A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Application of pre-treatments***																								
	1) Evaluation of different pre-treatments																								
	2) Application of pre-treatments on vegetable products																								
A2)	***Application of stabilization/transformation treatments***																								
	1) Different types of cooking																								
	2) Different conditions																								
A3)	***Evaluation of shelf life***																								
	1) Analysis during storage																								
A4)	***Thesis and Paper Preparation***																								

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Sustainable recovery of high value-added compounds from secondary raw materials of the milling industry

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The aim of this industrial PhD project is to valorise milling by-products from a nutritional and technological point of view. In particular, new products will be produced with technologically advanced methods starting from milling secondary raw materials in the innovative plant of Casillo Next Gen Food Srl, Corato (BA). These products will be nutritionally characterised, analysed for their rheological and technological properties and evaluated as possible ingredients for functional foods.

Recupero sostenibile di composti ad alto valore aggiunto da materie prime secondarie dell'industria molitoria

L'obiettivo di questo dottorato di ricerca industriale è capire come i sottoprodotti dell'industria molitoria possano essere valorizzati dal punto di vista nutrizionale e tecnologico. Nello specifico, nell'impianto innovativo di Casillo Next Gen Food Srl, Corato (BA), dagli scarti della molitura verranno realizzati nuovi prodotti con procedure tecnologicamente avanzate. Questi prodotti saranno caratterizzati dal punto di vista nutrizionale e tecnologico e valutati come possibili ingredienti per la formulazione di alimenti funzionali.

1. State-of-the-Art

Wheat grains are morphologically divided in three parts: endosperm, germ and bran. The endosperm accounts for 80-85 % of the kernel and is mainly constituted by starch. The germ makes up 2-3% of the kernel and is rich in lipids and vitamins. The wheat bran, made by the outer layers of the grains, provides protection to the whole kernel and is characterized by a high content of fibre and proteins. The milling operations aim is to remove the external layers of the grains and to collect the endosperm, to produce flour and semolina. Wheat germ, although particularly rich in micronutrients such as vitamins E and B, is generally excluded from the final products because oxidation of the lipids could affect the shelf life of the flour. Technological aspects, like the rheological behaviour of pasta and bakery products are also affected by the addition of dietary fibre above a certain amount, because of the interfering effect of non-gluten proteins that modify the consistency of dough gluten matrix (Hemdane et. al, 2016; Aravind et. al, 2012). However, considering that 20-25 % of the wheat kernel is constituted by bran and germ, a considerable amount of by-product is produced yearly by the milling industry and mostly used as feed in the livestock sector. After overcoming some technological problems, wheat by-products could gain a higher profile, especially given the benefits they can bring to human nutrition and in relation to the "zero waste" concept of agri-food promoted by the Farm to-Fork strategy of the European Green Deal (EU, 2020). The high fibre content of wheat bran helps to control postprandial glycaemic index, obesity and the incidence of type 2 diabetes (Prückler et. al, 2014), and also has prebiotic effects and promotes enteric equilibrium (Aravind et. al, 2012). However, not only fibre brings benefits for human health, but the whole set of bioactive/antioxidant compounds it contains (cellulose, lignin, arabinoxylans, polyphenols, etc.). The subsequent actions of separation and extraction of fibre and wheat germ components could allow to produce ingredients that mixed with flour or semolina could lead to obtain functional and non-functional foods. Reducing the particle size by micronization makes these products more suitable for transformation and incorporation into food. Moreover, the air classification process is able to change the distribution of nutrients by concentrating certain molecules in the fine and coarse fractions. In addition, purification of proteins and arabinoxylans (from bran and germ) through chemical or enzymatic methods can yield valuable isolates for further food applications. The aim of this PhD project is to follow the entire milling process in order to extract and to recover secondary raw materials, verify their properties (chemical, nutritional, functional, rheological) and apply physical enrichment/separation techniques to obtain innovative ingredients for the production of sustainable, healthy food.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Bibliographic research** for the continuous updating of knowledge on the specific topic.

A2) **Determination of chemical composition of secondary raw materials** from milling industry. The analysis

is meant to verify the health-promoting properties of the starting material before technological transformation.

A3) **Micronization and air-classification of secondary raw materials.** (A3.1) Operative setting of lab-scale micronization and turboseparation trials to produce fine and coarse fractions. (A3.2) Scale up of micronization and air-classification at industrial scale.

A4) **Characterization of the fractions obtained from air-classification.** (A4.1) Particle size analysis through laser diffractometry techniques. (A4.2) Evaluation of the presence of specific micronutrients (markers) attributable to the presence of bran or germ.

A5) **Rheological and technological evaluation of flours** from turboseparation system, also in mixtures with normal flour/semolina. (A5.1) Rheological properties analysis. (A5.2) Production on pilot plants, and then scale up to an industrial plant, of innovative food products (bread, pasta, etc.) and evaluation for their sensory, nutritional and technological attributes.

A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1)	***Bibliographic research***																		
A2)	***Determination of chemical composition of secondary raw materials***																		
A3)	***Micronization and air-classification of secondary raw materials***																		
	1) Lab scale																		
	2) Industrial scale																		
A4)	***Characterization of the fractions obtained from air-classification***																		
	1) Laser diffraction analysis																		
	2) Markers analysis																		
A5)	***Rheological and technological evaluation of flours***																		
	1) Rheological analysis																		
	2) Formulation, production and evaluation of innovative products																		
A6)	***Thesis and Paper Preparation***																		

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Development of vegetarian and vegan foods from lentil by-products for a healthy and sustainable diet

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The research project is developed within the "Partenariato Esteso" ON Foods Spoke 2 which aims to develop new foods through the use of innovative ingredients derived from agri-food by-products/wastes. This PhD program aims to characterise, design and develop high-value baked goods based on hull flour from red and green lentils with specific health and technological attributes. Physicochemical, nutritional, structural, and technological properties will be investigated to optimize the tailored foods for specific consumer populations.

Sviluppo di nuovi alimenti vegetariani e vegani da sottoprodotti della lenticchia per una dieta salutare e sostenibile

Questo progetto di ricerca, rientrante nell'ambito dello Spoke 2 Partenariato Esteso ON Foods, mira a sviluppare nuovi alimenti attraverso l'uso di ingredienti innovativi derivanti da sottoprodotti/rifiuti agroalimentari. In particolare, i principali obiettivi del progetto di dottorato sono la caratterizzazione e lo sviluppo di prodotti da forno ad alto valore aggiunto per specifiche popolazioni di consumatori. A tale scopo saranno dapprima testate le cuticole di lenticchie sia rosse che verdi poiché normalmente sono considerate uno scarto alimentare nonostante il loro interessante profilo alimentare. Lo studio della struttura e delle proprietà fisico-chimiche, termiche, funzionali e nutrizionali saranno effettuate per l'ottimizzazione dei prodotti finali.

1. State-of-the-Art

In the human diet, meat still serves as a significant protein source as in 2021, world meat production was estimated at 339 Mt, rising to 5% (OECD and FAO, 2022). Nevertheless, reducing meat consumption is advised due to increasing apprehensions about the environmental consequences of livestock production and the health hazards associated with excessive meat intake. Consequently, there is a growing requirement for plant-based diets and more efficient methods of food processing to address these concerns (Monnet *et al*., 2019; Young and Pellett, 1994). Legumes are consumed worldwide and are desired for their high protein quality and quantity. In particular, lentils (*Lens culinaris Medikus*) are a pulse crop belonging to the Fabaceae family and are grown in more than 70 countries, ranking fourth in the global grain legumes production after bean (*Phaseolus vulgaris L.*), pea (*Pisum sativum L.*) and chickpea (*Cicer arietinum L.*) (Kumar and Pandey, 2020). Lentil cotyledon is lens-shaped and may have a wide range of colours (yellow, orange, red or green), even though the most traded classes are red and green (Romano *et al*., 2021). Lentils are nutritionally beneficial to all, including vegetarian and vegan diets. Lentils are usually used for consumption in the form of cooked whole seeds, split cotyledons or processed into various ingredients (e.g., flour) for use in different food applications (Romano *et al*., 2021). Due to consumer preferences, dehulling is a common procedure for most lentil market classes. Pulse seed hulls are primarily used as low-value animal feed, with limited applications in human foods such as high-fibre bread and meat products. This by-product poses a disposal problem for millers, while it could potentially be a source of novel, nutritious, and health-promoting food ingredients. (Sherasia *et al*., 2017). Indeed, hulls are rich in phenolic compounds, dietary fibre, and phytochemicals that have anti-inflammatory, antioxidant, lower blood pressure, cholesterol and blood sugar properties (Dueñas *et al*., 2006). Hence, it would be ideal to recover the hulls, as they have a technological and nutritional potential, which would otherwise be lost as they are considered a waste product of food industries. They could be studied for application in various product formulations, including bakery (bread, cake, crackers) and extruded (pasta, snacks). However, there are various anti-nutritional factors (ANFs) in lentil hulls including lectins, α-amylase inhibitors, protease inhibitors, phytic acid and tannins that limit their extensive usage in food industries. Thankfully, various post-harvest operations and processing techniques have been shown to reduce ANFs. These include heat treatments such as wet and dry heating, steaming, boiling, and extrusion (Sharma *et al*., 2022).
Among the seven thematic spokes of the PNRR ON FOODS project lies the valorisation of waste and by-products from the food industry. In particular, Spoke 2 aims to improve the sustainability of food systems through circular economy processes for the recovery of by-products to obtain high-added value products.
Hence this PhD project aspires to investigate the safety, nutritional value and properties of a new food enriched with flour obtained from red and green lentil hulls. This new ingredient will be characterized using physicochemical and innovative spectroscopic analyses (e.g., NIR, NMR). The potential use of lentil hulls as an ingredient in bakery products will be tested. To optimize the products and processes, the structure (macro and

micro), physicochemical, functional and nutritional (e.g., ANFs and glycemic index) properties of trials made will be studied. Once the new foods have been characterised, the aim would be to evaluate the in vitro gastrointestinal digestion of the new products and the behaviour of certain constituents with high biological activity once inside the human body.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Evaluation of microstructure and physicochemical, functional, thermal and nutritional properties of lentil hulls.**
A2) **Evaluation of the most suitable spectroscopic technique (e.g., NIR, NMR) for characterising the lentil hull ingredient**.
A3) **Evaluation of potential application in the food industry**.
A4) **Characterization of the properties of the dough.** The doughs obtained with different percentages of flour samples will be subjected to analysis for determination of physicochemical and rheological properties.
A5) **Evaluation of the impact of hull flour on baked products.** The influence of flour on the formulations of bakery products will be studied by analysing their properties and *in vitro* digestibility.
A6) **Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.**

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Evaluation of properties of lentil hulls***																								
	Microstructure																								
	Physicochemical, nutritional, thermal and functional analyses																								
A2)	***Evaluation of the most suitable spectroscopic technique for characterising the lentil hulls ingredient***																								
	Spectroscopic analyses (NIR, NMR)																								
A3)	***Evaluation of potential application in the food industry***																								
	Literature analysis																								
A4)	***Characterization of the properties of the dough***																								
A5)	***Evaluation of the impact of hulls flour on baked products***																								
	Microstructure (SEM and CLMS) and macrostructure (Image Analysis protocol)																								
	Physicochemical, functional, nutritional and rheological properties																								
	In vitro digestibility																								
A6)	***Thesis and Paper Preparation***																								

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Use of genetic and genomic resources to improve nutritional quality and shelf-life in pepper.

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This PhD thesis project aims at the preparation of experimental mapping populations including recombinant crossing populations to be used for the dissection of the genetic bases of complex traits responsible for pepper quality. Shelf-life and content of antioxidant compounds will be the focus traits to be investigated. The project aims not only to provide tools for basic research but also for qualitative and quantitative genetic improvement by bridging the gap that currently exists with respect to other agricultural crops (e.g., cereals, tomatoes).

Utilizzo di risorse genetiche e genomiche per migliorare la qualità nutrizionale e la *shelf-life* in peperone.

Questo progetto di tesi di dottorato basato mira allo sviluppo di popolazioni sperimentali quai collezioni "*core"* di germoplasma e di popolazioni ricombinanti ottenute mediante attività di inter-incrocio da utilizzare per lo studio delle basi genetiche di caratteri complessi responsabili della qualità in peperone. *Shelf-life* e contenuto in sostanze antiossidanti saranno i principali caratteri da studiare Il progetto mira non solo a fornire strumenti per la ricerca di base ma anche per il miglioramento genetico quali-quantitativo colmando il divario ad oggi esistente rispetto altre colture agrarie (es. cereali, pomodoro).

1. State-of-the-Art

Climate change and its consequences are emerging as one of the main challenges to deal in the near future. Agriculture is the sector most affected by these changes, therefore, the increase in agricultural production together with the stability of production and the quality of products represent the crucial objective for the economy and food security of all countries. Pepper (*Capsicum* spp.) is an important member of the Solanaceae family, it is a main vegetable and spice crop originated in the American tropics and today cultivated all over the world for fresh, dried, and processing products (Patel et al., 2019). According to recent estimates there are more than 35 pepper species grouped in 11 clades (or complexes), three of which (Annum, Baccatum and Pubescens) encompass domesticated and wilds relevant in terms of nutritional and economic importance and widely used for genetic improvement. Around the genus *Capsicum* there is an increasing interest and fascination due to the considerable variation for several traits, which makes this crop extremely versatile and suitable for innumerable uses as food and non-food products. *Capsicum* fruits are highly rich in pharmacological compounds such as carotenoids (provitamin A), vitamin C and E, flavonoids and the distinct metabolite alkaloid complex known as capsaicinoids which impart pungency to its fruits. In recent years, several genomes of domesticated and wild *Capsicum* species have been sequenced, which provided a basic infrastructure for subsequent genomic studies (Ziv et al.,2022). High yield, early flowering, biotic and abiotic stress tolerance, enriched metabolite content, desired fruit size and shape and reduced postharvest water loss have been major targets for pepper improvement mostly by classical breeding efforts. Marker-assisted selection and genome-wide association studies for the useful exploitation of resistance genes and QTLs have offered considerable advantages over the conventional plant breeding approaches for the improvement of *Capsicum* in terms of accuracy, specificity, and duration (Uffelmann et al., 2021). Furthermore, the next-generation sequencing technologies have proven breakthroughs in the field of identification of the genomic regions responsible for stress tolerance, evasion and responses which could be employed for future *Capsicum* breeding programs.

In pepper it is estimated that there are over 30 thousand accessions available in the network of international gene banks (Barchenger et al., 2022); the existing germplasm represents an important source of characteristics of interest

for agriculture, which, however, at present is still little used for genetic improvement. The innovation of the project concerns the development of advanced germplasm resources through breeding and selection activities and the enrichment with genomic information obtained through latest generation sequencing technologies and phenomic characterizations for the main characteristics required by the market and consumers. These activities allow to define potential germplasm platforms to be used for the discovery of new key genes.

2. PhD Thesis Objectives and Milestones

The PhD thesis project, within the general objective mentioned above, can be divided into the following phases according to the Gantt diagram given in Table 1:

A1) **Development of experimental populations** including F_2 and Backcrosses derived from intra- and inter-specific crosses and core germplasm set.

A2) **Application of *next generation sequencing* for genomic characterization** through ddRAD-seq (Double digest restriction-site associated sequencing).

A3) **Phenotyping** for shelf life an main metabolites underlying pepper quality (vitamin C, flavonoids, carotenoids).

A4) **Scanning genomic regions underlying phenotypes** through *quantitative trait loci mapping* **(QTL***)* and genome wide association approaches.

A5) **Development of functional genomic markers**, developed for precision breeding and assisted selection of target genes.

A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1: Gantt diagram for this PhD thesis project.

Activity	Trimester	1	2	3	4	5	6	7	8	9	10	11	12
A1)	Mapping populations	■	■	■									
A2)	Genomic characterization			■	■	■							
	1) Nucleic acid isolation			■									
	2) ddRAD sequencing				■	■							
A3)	Phenotyping			■	■	■	■	■	■				
	1) Shelf life				■	■							
	2) Metabolic compounds				■	■	■	■	■				
A4)	Gene mapping							■	■	■	■	■	
A5)	Functional markers										■	■	
A5)	Thesis and Paper Preparation	■	■	■	■	■	■	■	■	■	■	■	■

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From agri-food waste to high-value compounds via green technology approaches

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The aim of this PhD project regards the use of green technologies such as biocatalysis, enzyme immobilization, and "in continuous" processes to transform molecules or extracts derived from agri-food waste in natural valuable compounds/enriched extracts with attractive biological properties (circular economy approach). The combination of the above cited technologies demonstrated to enhance the sustainability, selectivity and productivity, of chemical processes.

Dai rifiuti agoalimetari ai composti ad alto valore attraverso approcci di green technologies

L'obiettivo di questo progetto di dottorato riguarda l'uso di tecnologie green come biocatalisi, immobilizzazione enzimatica e processi "in continuo" per trasformare molecole o estratti derivati da scarti agroalimentari in composti naturali di pregio/estratti arricchiti con interessanti proprietà biologiche (approccio di economia circolare). È stato dimostrato che la combinazione delle tecnologie sopra citate migliora la sostenibilità, la selettività e la produttività dei processi chimici.

1. State-of-the-Art

With the population growth approaching now 8 billion people as well as the modification of the life-style and the eating habits, a large increase of the agri-food companies involved in the production and processing of food-related compounds was observed, so much that agri-food-industrial waste became a significant environmental and economic problem (Sagar *et al*., 2018). In fact, tones of agri-food residues generally made up of seeds, peels, leaves, skins, branches, trunks, roots are typically unexploited and need to be managed and disposed. Moreover, most of these residues have demonstrated to contain natural valuable compounds with attractive biological properties. In this context, waste minimization together with the possibility of bioactive recovery and their modification through green methodologies can represent an appealing business opportunity for the agri-food, nutraceutical, cosmetic and pharmaceutical companies, among others (Tonini *et al*., 2018).
Biocatalysis, the branch of biotechnologies aiming at using living systems or their parts (enzymes) to catalyze chemical reactions has been recognized as a valuable tool for chemists if stable and robust biocatalysts can be employed (Contente *et al*., 2021). In this context, "extremozymes" proteins derived from microorganisms adapted to live in drastic environments (e.g., high/low temperature solvent and pH) demonstrated to be more tolerant to industrial processes with respect to the mesophilic counterparts.
To further increase enzyme stability and allow their recover and reuse, immobilization techniques on solid supports can be exploited, also facilitating catalyst incorporation in flow chemistry reactors (Bommarius *et al*., 2013).
Among the advantages of "in continuous" systems high local concentration of the biocatalyst, superior mass and heat transfer, impacting on reaction times as well as the addition of in-line work-up and purification steps enhancing the system automation, are noteworthy. Moreover by adding more bioreactors in series it is possible to set up multi-enzymatic cascade reactions (Benítez-Mateos *et al*., 2021).
In this project we decided to focus our attention on natural glycosides and their transformation into the corresponding aglycones characterized by superior bioavailability and/or bioactivity. To do this extremophilic β-glycosidases were selected as biocatalysts together with four different agri-food residues as glycosides natural sources.
Soybean cultivation produces one of the largest agri-waste worldwide. The residues contain three types of isoflavones (daidzein, genistein, and glycitein), which can be found mainly as glycosides. Recently, commercial preparations of isoflavones have come to the public attention due to their positive effects on cognitive function. However, when the biological activities of these compounds are considered, the bioavailability of the aglycones has been suggested to be higher than that of the glycosides; but they represent only a minor constituent of soy-residues. B-glycosidases can be used to hydrolyze isoflavone glycosides to their aglycones, thus obtaining high-value products. Among natural fragrances, vanilla is the most employed in the perfume, cosmetic and food industry, due to its aromatic characteristics. Being its plant extraction quite expensive, several synthetic strategies have been developed for vanillin preparation. As an important fraction of vanillin is still present as glucovanillin in the plant residues as well as in the wastewater distillation, β-glycosidases can be used to hydrolyze this glycoside to its

aglycone.
Enzymes can also be associated in cascade reactions. For example for the preparation of hydroxytyrosol one of the most powerful antioxidants starting from oleuropein, the major component of olive leaves and branches as well as for the obtainment of the aglycones from citrus rutinosides (e.g., rutin and hesperetin) mainly present in the inedible parts of citrus fruits, especially peels and leaves.
According to FDA, EMA and EFSA regulation, processing natural molecules through biocatalytic approaches allows for the final compounds to be claimed as natural, thus increasing their market value.

2. PhD Thesis Objectives and Milestones

Table 1 *Gantt diagram for this PhD thesis project.*

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1	***Glycoside green extraction, purification, and analysis***																								
A2	***Batch reaction optimization***																								
A3	***Enzyme immobilization***																								
A4	***Flow processing***																								
A5	***Thesis and Paper Preparation***																								

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Glycoside green extraction, purification, and analysis.** Green methodologies involving enzyme-ultrasound- and microwave-assisted extractions will be employed. Green solvents, ionic liquids (IL), deep eutectic solvents (DES), or non-polar GRAS solvents such as 2-methyl tetrahydrofuran (2-MeTHF), D-limonene, ethyl acetate will be investigated in order to obtain safer, eco-friendly and more efficient extractions (Gullon *et al*., 2020; Chemat *et al*., 2019). Identification and quantification of the desired glycosides will be carried out via HPLC/GC analysis. This part will be performed in collaboration with partners with strong expertise in the field of green extraction and analysis.

A2) **Batch reaction optimization.** Single-step and one-pot, multi-step reactions will be firstly optimized in batch mode. The obtained results will be fundamental to understand the catalyst compatibility and to select the best immobilization strategy.

A3) **Enzyme immobilization.** Catalyst stability will be improved via enzymatic immobilization techniques. Particular attention will be paid on a covalent bond between the protein and methacrylate or agarose microbeads demonstrated to be the most suitable carriers for "in continuous" operations. The best-performing enzymes will be immobilized and assayed for operational stability and reusability.

A4) **Flow processing.** In-continuous processes will be developed to increase the productivity and sustainability of the process. To solve solubility problems, water-miscible co-solvents, multi-phase enzymatic reactions, or pure organic solvents will be studied. In-line extractions and purification protocols will be carried out, thus avoiding any manual handling and increasing the safety and automation of the system.

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

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Evaluation, characterization and conservation of microbial diversity in complex ecosystems of agri-food interest

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This PhD thesis project aims at developing long-term storage protocols to preserve the taxonomic stability, cell viability and metabolic activity of complex microbial communities (microbiomes) isolated from food matrices. In addition, the isolated microbial species, and the associated genetic and metabolic information, will be functional for the implementation of the Microbial Collection of the University of Sassari (MBDS-UNISS-CC). The PhD research work is carried out within the framework of the activities of the SUS-MIRRI project 'Strengthening the MIRRI Italian Research Infrastructure for Sustainable Bioscience and Bioeconomy' and the technical-scientific collaboration agreement within the 'Hermàion 2.0' project.

Valutazione, caratterizzazione e conservazione della diversità microbica in ecosistemi complessi di interesse agroalimentare

Questo progetto di tesi di dottorato mira alla messa a punto di un protocollo sperimentale atto a garantire la conservazione, la stabilità e la vitalità di comunità microbiche complesse da matrici alimentari. Le specie microbiche isolate e le informazioni ad esse associate andranno ad incrementare la Collezione Microbica dell'Università di Sassari (MBDS-UNISS-CC) e il relativo Database. Il lavoro di ricerca del dottorato si svolge nell'ambito delle attività del progetto SUS-MIRRI "Strengthening the MIRRI Italian Research Infrastructure for Sustainable Bioscience and Bioeconomy" e dell'accordo di collaborazione tecnicoscientifica nell'ambito del progetto "Hermàion 2.0".

1. State-of-the-Art

Microbial resources play a central role in biotechnological innovations, and their use in food, industry, medicine, environment and agriculture has been widely discussed in the literature. To meet the growing demand for fermented foods, the use of microbial starters ensure that high quality standards are achieved and maintained. However, their control over the microbiota naturally associated with the raw material can lead to a significant reduction of biodiversity and sensorial complexity of the final products. Indeed, it is now widely accepted that microbial communities should be considered as a whole, as the activity of a single component is modified and regulated by those of other community members. Microbial communities, considered as dynamic ecological niches, are central to food microbiologists, especially when considering their evolution and fluctuations during the fermentation processes. The number and diversity of microorganisms in fermented foods and drinks varies according to the geographical area, climatic factors, environment, raw materials used and their preparation methods (M.Walsh et al.,2023). Therefore, the use of complex microbial communities in traditional fermentation processes (e.g sourdough) has become increasingly important. In addition, the use of traditional microbial communities combined with modern production processes leads to the production of new fermented foods and drinks enriched in taste and aroma (Giraffa, 2004). A key point in the availability of complex microbial starters for scientific and industrial applications is to assure the long-term maintenance of their taxonomic and metabolic characteristics. The conservation of complex microbial communities is thus a challenge for microbiologists, due to the lack of standardized and effective storage programs to optimally preserve both tolerant and sensitive microorganisms. This is of particular importance for Culture collections (CCs) and Microbial Resource Centers (CRMs), which currently base their activities on the conservation and maintenance of microorganisms mainly in pure culture. Several techniques are widely used to preserve microbial resources ex situ (De Vero et al., 2019). According to the guidelines of the World Culture Collection (WFCC) and Organization for Economic Cooperation and Development (OECD), at least two techniques should be used to maintain each microbial culture. Particularly, cryopreservation and lyophilization are the most reliable and widely used methods for long-term preservation of many biological resources. Also, to reduce the risk of accidental loss, additional storage should be established in different places on site and preferably with different storage techniques to ensure the long-term preservation of the harvest (Prakash et al., 2013). In this context, the stability of taxonomic, genetic, metabolic and functional characteristics of complex microbial communities in food matrices will be determined by means of NGS techniques, Phenotype microarray, RNA-seq, following different community preservation methods.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Validation of SOPs for the sampling of microbiome in different ecosystems** (Definition and validation of Standard Operation Procedures (SOPs) for the sampling of microbiomes from different sources (A1.1, A1.2)).

A2) **Test performance study for the microbiome analysis** (SOPs for genetic characterisation of microbiomes with Hig-throughput sequencing (HTS) technologies, including sample preparation, sequencing protocol, databases and bioinformatic pipelines).

A3) **Optimization of analytical methods to follow the quality of microbiomes during storage** (Intermediate results of the optimization of analytical methods for quality and compliance of the microbiomes (A3.1)).

A4) **Definition of conditions and protocols for long-term preservation of microbiomes** (Optimisation of suitable methods to control the quality of the microbiomes during storage (after 6 and 12 month) (A4.1, A4.3). Definition of the best conditions to safely store microbiomes. Design of appropriate methodologies to propagate the conserved microbiome prior to utilisation and proof of concept related to safe deposit of microbiomes (A4.2).

A5) **Application of the knowledge acquired to safely store and reutilize microbiomes from different sources.**

A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 *Gantt diagram for this PhD thesis project.*

Activity	Month	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
A1)	*Validation of SOPs for the sampling of microbiome in different ecosystems*																														
	1) SOPs for the analysis of microbiome in different ecosystems																														
	2) Defininiition of the SOPs																														
A2)	*Test performance study for the microbiome analysis*																														
A3)	*Optimization of analytical methods to follow the quality of microbiomes during storage*																														
	1) Intermediate results of the optimization of analytical methods for quality and compliance of the microbiomes																														
A4)	*Definition of conditions and protocols for long-term preservation of microbiomes*																														
	1) Best conditions for the storage of the microbiome (month 6) as determined by comparing the results obtained with those of the microbiome at the beginning of the storage																														
	2) Best propagation approach to guarantee compliance with the initial microbiome stored																														
	3) Best conditions for the storage of the microbiome (month 12) as determined by comparing the results obtained with those of the microbiome at the beginning of the storage																														
A5)	*Application of the knowledge acquired to safely store and reutilize microbiomes from different sources*																														
	1) Preliminary results on the application of the stored microbiomes in different sources																														
A6)	*Thesis and Paper preparation*																														

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Investigation of spontaneously fermented food matrices using high throughput DNA sequence-based analytical methods to explore microbial ecology

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This PhD thesis research project is aimed at investigating the drivers of a spontaneously fermented food matrix (e.g., coffee, cocoa, and table olives) through a multi-omics approach. The use of high throughput DNA sequence-based analytical methods will contribute to exploring microbial ecology and will be used as an indicator of origin to study the authenticity of raw materials.

Indagine su matrici alimentari fermentate spontaneamente utilizzando metodi analitici ad alto rendimento per il sequenziamento del DNA per esplorare l'ecologia microbica

Questo progetto di tesi di dottorato mira a studiare diverse matrici alimentari fermentate spontaneamente (es. caffè, cacao e olive da tavola) per mezzo di un approccio multi-omico. L'utilizzo di metodi di sequenziamento ad alto rendimento contribuirà ad esplorare l'ecologia microbica in modo da utilizzarla come indicatore di origine per studiare l'autenticità delle materie prime.

1. State-of-the-Art

The microbial ecology of food concerns the study of the type of microorganisms present (diversity and population number), their rate of occurrence, activities (functionality), and interactions with each other (microbial communities) in the environment. In recent years, the study of the microbiome has become increasingly important to understand the interactions between microbial populations, especially in fermented foods as they are a complex matrix (De Filippis et al., 2018). Both culture-dependent and independent methods are essential for investigating food microbial ecology. However, it is estimated that 99% of microorganisms present in nature are typically not cultivated using standard laboratory techniques (Amann et al., 1995). The application of molecular methods has allowed the detection of populations that were largely undetected previously, thus generating a more accurate definition of the drivers involved in the fermentation process and their functions (Cocolin et al., 2013).
Cocoa and coffee beans have a complex microbial ecology that has a crucial role in the sensory characteristics respectively of chocolate and coffee because aroma and flavor precursor molecules are synthesized during the fermentation process. Thus, diversity could derive from yeast and bacterial populations present in the close environment during this process. When the fermentation occurs in a spontaneous or uncontrolled manner, the chemical and sensory quality of beans varies from location to location and can be compromised because of the non-controlled process. Hence, the variability of the fermenting microbial species from different regions remains one of the main factors influencing the variability of products' quality (Figueroa-Hernandez et al., 2019).
Table olives, instead, are produced from the raw drupe fruits deriving from varieties of the cultivated olive tree. The raw fruit is inedible and highly bitter due to the presence of oleuropein, which needs to be degraded. Treatments to reduce the bitterness of the fruit involve brining, acidification, enzymatic hydrolysis, and spontaneous fermentation (Perpetuini et al., 2020). During these processes, the microbial ecology varies and may originate from the raw materials and the environment, in which it is possible to detect mixed yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB), filamentous fungi, spoilage bacteria, and others (Serra et al., 2019).
The spontaneous process for cocoa and coffee involves yeasts that belong to the Saccharomycetaceae family including Hanseniaspora, Saccharomyces, Kluyveromyces, and Pichia that can produce ethanol from the fermentation of carbohydrates and can be responsible for flavour precursor production, with a concomitant increase in temperature. Lactic and acetic bacteria groups (e.g., Limosilactobacillus fermentum, Lactiplantibacillus plantarum, and Acetobacter pasterianus) are responsible for lactic acid and acetic acid production (Figueroa-Hernandez et al., 2019). In the case of table olives LAB (e.g., Lactiplantibacillus plantarum, Lactiplantibacillus pentosus) are responsible for oleuropein degradation thanks to their enzymatic activities and cause the acidification of the final product, providing microbial stability and elongating the shelf-life of the olives while yeasts (e.g., Saccharomyces cerevisiae, Candida boidinii) are involved in the production of volatile compounds and metabolites that increase the overall quality and preserve their sensory features (Perpetuini et al., 2020).
Next-generation sequencing (NGS) is fundamental to obtain a deeper and more detailed characterization of the

microbiome. In amplicon sequencing, template DNA is fragmented, bound to a substrate, and amplified by PCR to produce clonal representations of the original spatially separated fragments for subsequent sequencing, while shotgun sequencing requires genomes to be broken into small fragments, sequenced, and reassembled using overlapping sequences (Bedia, 2018). These techniques are used to describe in great detail microbial communities' structure and function and the information deduced is used as an indicator of origin to study the authenticity of raw materials.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1.

A1) **Literature review and experimental design definition**

A2) **Optimization of sample preparation and DNA extraction protocols compatible with high-throughput sequencing** to study the entire microbial ecology (yeasts and bacteria) of the different matrices. The protocols will be tested to understand which method will be useful for subsequent Next Generation Sequencing to obtain a deeper and more detailed characterization of the microbial ecology.

A3) **Sequencing and bioinformatics analysis** to investigate 16S, and 26S rRNA genes amplicon (metataxonomy) on a large number of samples. The data will be analyzed using bioinformatics tools to obtain the entire microbial ecology of the different matrices and to understand the differences between the origins.

A4) **Metabolome analysis** using gas chromatography-mass spectrometry (GC–MS), and liquid chromatography-mass spectrometry (LC-MS) to understand if the different origins of the matrices will also influence the production of secondary compounds and thus the final quality of the product.

A5) **Marker analysis for safety, quality, and authenticity,** correlating microbiological and metabolite trends with the origin of the different matrices. Finally, links between microbial ecology, metabolites, and abiotic factors (e.g., climatic parameters, interventions in primary production, and local practices) will be investigated to identify markers of safety, quality, and authenticity.

A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	*Literature review and experimental design definition*	■	■																						
A2)	*Optimization of sample preparation and DNA extraction protocols compatible with high-throughput sequencing*	■	■	■	■	■	■	■	■																
A3)	*Sequencing and bioinformatics analysis*				■	■	■	■	■	■	■	■	■	■	■										
A4)	*Metabolome analysis*													■	■	■	■	■	■	■					
A5)	*Marker analysis for safety, quality, and authenticity*													■	■	■	■	■	■	■	■	■	■		
A6)	*Thesis and Paper Preparation*	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

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Agri-food industry by-products valorisation: a focus on pomegranate peel extracts used as a tannin-rich ingredient in different food areas, including winemaking

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Pomegranate peel, a by-product from juice production, is a rich source in bioactive compounds. Extraction will be performed with different green methodologies based on the type of application. Both the tannin and the pectic fraction of the extracts will be chemically characterized. Their addition in baked goods and their use as oenotannin in winemaking will be tested, to evaluate technological and antioxidant properties. All enriched/fortified products will be subjected to sensory analysis to determinate any variations after the addition of extracts.

Valorizzazione di un co-prodotto dell'industria agro-alimentare: focus su estratti di buccia di melagrana utilizzati come ingredienti ricchi di tannini in differenti settori alimentari, inclusa la vinificazione

Le bucce di melograno, co-prodotti derivanti dalla produzione di succo, sono una risorsa ricca di composti bioattivi. L'estrazione delle bucce avverrà con diversi metodi green a seconda del tipo di applicazione dell'estratto. Questi verranno caratterizzati rispetto alla frazione fenolica e polisaccaridica. Si testerà la loro aggiunta in prodotti da forno e come tannino enologico nel vino, per valutarne al meglio le proprietà tecnologiche e antiossidanti. Tutti i prodotti addizionati saranno soggetti a test sensoriali per valutare variazioni dopo l'aggiunta degli estratti.

1. State-of-Art

Pomegranate is receiving an increased attention because of its abundance in bioactive compounds with beneficial health properties. Since it was defined by the media as "superfruit", its demand had a significant spike, followed by an increased demand of pomegranate-derived products such as juices, jams, flavored water and salad/dessert dressings (Kahramanoglu, 2019). Peel counts for about 50% of the fruit weight, and the production of this waste is estimated in ≈ 1.9 million metric tons in 2017. This waste needs to be address to other types of production because, if put in landfills, it represents a threat for the environment. It could be used as fertilizer, bio-adsorbent or animal feed as such, but since the peel is rich in valuable compounds (Valero-Mendoza *et al.*, 2023), studies on nutraceutical-technological uses are ongoing. Innovative extraction strategies are required, toward more sustainable procedures of by-product treatment. Hydrodynamic cavitation (HC) is an eco-friendly and cost-effective extractive technology, which allows good yields and easy scale-up of the process. It is based on the phenomenon of cavitation, which occurs when negative pressure is applied to a liquid, where small cavities (microbubbles) filled with gas are formed. When the pressure rises up again, bubbles collapse generating a local shock wave that disrupt the plant matrix in that area, causing extraction. This technique, not tested so far on pomegranate, is reported to well extract the pectin fraction from matrices (Presentato *et al.*, 2020). Also, low temperature, solid/liquid extraction will be performed, to exclusively extract the tannin fraction. This technique, based on the mass transfer process (diffusion) from a high concentration area to a low concentration area, allows a higher selectivity towards target compounds due to solvent selection and temperature control (Vorobiev and Lebovka, 2020). Maximization of phenols and absence of polysaccharides will be achieved with hydroalcoholic media and low temperatures, since polysaccharides are not soluble in these conditions.

1.1 Pectin substances in pomegranate and possible applications

Pomegranate peel could be considered a good source of polysaccharides, with crude fibers being accounting for about 21% of the peel and total carbohydrate the 86% (Al-Rawahi *et al.*, 2013). Polysaccharides of pomegranate are part of the dietary fibers and are mostly constituted by pectin material, but different extraction methods could bring to different yields and quality of the pectin. Key extraction parameters are extraction time, extraction temperature, DM/solvent ratio and pH. Health benefits of pomegranate pectin have been evaluated *in vitro* because soluble fibers are well-known to explicate a prebiotic effect; in fact, *B. breve* B632 and *L. plantarum* L12 strains grew well on pomegranate polysaccharides as a carbon source, in a comparable way to the strains fed on glucose (Khatib *et al.*, 2017). Moreover, pectin has important technological properties, such as thickening and gelling properties. To practically evaluate these aspects, the extract will be inserted in a bakery product, in substitution to the elements of the recipe that give structure to the final product, flour and sugar.

1.2 Tannins in pomegranate and possible applications

Pomegranate tannins are the most studied portion of the peel, responsible for antioxidants, antimicrobial and antiviral properties. Other health benefits are anti-inflammatory, anti-allergenic, anti-diabetic and anti-hyperlipidemic properties (Valero-Mendoza *et al.*, 2023). The most important class of tannins found are ellagitannins (hydrolysable), with some particular compounds unique of pomegranate, such as punicalagins and

punicalins. Area of applications of pomegranate tannin-rich extract are numerous, from pharmaceutical and nutraceutical ingredients to food preservatives, food colorant, bio-stimulant for plant growth (Pathak, Mandavgane and Kulkarni, 2017).Innovative solutions of application are as additive in edible coatings (Salem *et al.*, 2022) and as oenotannin in wine-making (Canuti *et al.*, 2020). To further analyze the possible use of pomegranate tannin in enology, the characterized extract will be tested in different wine-making conditions.

2. Objective

Objective of this study is pomegranate peel valorization. The structure of the research and the different aims are:

1) **Application of green, innovative extraction methods**: hydrodynamic cavitation and low temperature alcoholic solid/liquid extraction. The derived extracts will be chemically characterized. Tannin fraction will be evaluated through HPLC-DAD-MS and pectin fraction through SEC, DLS, ^{1}H-NMR analysis and HPAEC-PAD for sugar analysis.
2) **Application of the hydrodynamic cavitated extract in the bakery sector**: the use as thickening agent/sugar substitute in a vegan-gluten free product will be tested. Structural properties (weight, spread ratio, water activity, total humidity, color, and texture) will be also evaluated as well as sensory and antioxidant (DPPH and Folin-Ciocalteau assays) properties. Changes of these properties during time will be evaluated.
3) **Application of the alcoholic extract in the wine-making sector**: the use as oenotannin in red and white vinification will be tested. On wines will be conducted the following analysis at different times after bottling: color intensity, hue, monomeric anthocyanins content (HPLC-DAD), polymeric colored pigment (HPLC), total phenols (HPLC), anti-radical activity through DPPH assay, sensory evaluation.
4) **Data elaboration and PhD thesis writing**

Timing of he mentioned activities is proposed in table 1, with the Gantt diagram.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Extraction and characterization***																								
	1) HC extract																								
	2) Alcoholic extract																								
A2)	***Bakery application***																								
	1) Analysis of bakery product																								
	2) Sensory evaluation																								
A3)	***Wine-making application***																								
	1) Preparation of wines																								
	2) Analysis of wines																								
	3) Sensory evaluation																								
A5)	***Thesis and Paper Preparation***																								

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Methodologies to guide consumers towards a healthy and sustainable Mediterranean Diet

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This PhD thesis research project aims to identify innovative strategies to increase awareness regarding the health and environmental benefits of a high adherence to the Mediterranean Diet (MD) and improve healthy food choices in the daily lives of young people and their families.

Metodologie per accompagnare i consumatori verso una Dieta Mediterranea sana e sostenibile

Il presente progetto di tesi di dottorato si propone di identificare e sviluppare strategie innovative per incrementare la consapevolezza in merito ai benefici per la salute e per l'ambiente che possono derivare da un'elevata aderenza alla Dieta Mediterranea (DM) e da scelte alimentari sane nella vita quotidiana dei giovani e delle loro famiglie.

1. State-of-the-Art

Over the past 50 years, improved food systems have led to an increase in life expectancy and reductions in infant mortality, hunger, and global poverty. However, nowadays a global shift towards unhealthy diets is contributing to an increase in obesity and non-communicable diseases (NCDs), as well as environmental degradation and depletion of natural resources (Willett *et al*., 2019). Focusing on the Mediterranean region, this problem concerns the adherence to the Mediterranean Diet (MD), which is moderate to low (Obeid *et al*., 2022). This may be attributed to the increasing process of globalisation and urbanisation, the increase in incomes, the diffusion of supermarkets, the change in family structures, and the development of mass food culture (FAO and WHO, 2019). Fortunately, the scientific evidence on the benefits of the MD is well known and the urgent need to reverse current trends is increasingly stressed. Indeed, it has been evidenced that a high adherence to MD prevents cardiovascular events, improves lipid profile and adiposity levels, and reduces the risk of overweight and obesity, metabolic syndrome (MetS), and type 2 diabetes mellitus (DMT2) in adults (Seral-Cortes *et al*., 2022). Moreover, according to FAO, following this diet can reduce environmental impacts (FAO and WHO, 2019). In fact, as shown in the "Double Food and Environmental Pyramid" developed by the Barilla Center for Food and Nutrition, the MD (based on a high consumption of vegetables, fruits, nuts, unrefined grain cereals, with some fish and limited amounts of red meat and saturated fats) matches with the environmental pyramid thanks to an inverse relationship between nutritionally recommended foods and their environmental impact (Ruini *et al*., 2015). People, particularly the young generation, seem to show a growing interest in a healthy and sustainable lifestyle, albeit knowledge about nutrition and the meaning of sustainability are still poorly understood. In addition, the increasing use of different information channels and social networks complicates the context since the information conveyed is not always reliable.

Therefore, tackling the rapid evolution of this declining adherence to high-quality diets requires timely action at multiple levels of intervention targeting both food production and final consumption. Concerning consumers, nutritional interventions based on nutrition education could be a winning strategy, especially in the young population. Nutrition education should be provided at an early stage, to establish correct and sustainable eating habits in children that can persist over time, to achieve benefits not only in the present but also in the long term. Thus, educational interventions for the whole family would be more effective than communication with adults alone. Based on these considerations, addressing different age groups implies different educational efforts involving schools, national programmes, and the media (Willett *et al*., 2019). In addition, among the traditional tools, dietary guidelines, labels, and infographics are the most used. More innovative methods are also becoming more widespread, such as the use of web applications to improve diet and monitor nutrition (Schoeppe *et al*., 2016). A learning-through-playing approach has been also gaining attention over the last few years as an efficient strategy (Rosi *et al*., 2016). Also worth mentioning is the possibility of promoting healthy snacks to switch on the interest about healthy choices that are often consider more boring and not tasty. Thus, this last strategy involves a close cooperation with food production, which should develop and promote new healthier products more appealing for consumers. Consequently, so far, the opportunities to convey nutrition education are numerous, but it is necessary to further explore this area to implement and combine existing methodologies to develop new ones, more efficient and successful.

Based on these considerations, the aim of this PhD thesis research project is to investigate new methodologies to foster nutrition education, with a particular focus on families.

2. PhD Thesis Objectives and Milestones

The following steps can be considered for this PhD thesis project according to the Gantt diagram in Table 1:

A1) **Consumer science and co-creation**: sample populations (children, adolescents, adults) from different Mediterranean region are selected and surveyed on several areas: adherence to the Mediterranean Diet (barriers and drivers), food preferences and habits, nutritional knowledge, and general lifestyle. The information tools and channels through which the subjects learn about nutrition are also investigated to understand what strategies are currently applied, their potential and their limitations. The investigation is also implemented by conducting different focus groups.

A2) **Development of innovative educational strategies and other tools**: from the collected data, new intervention strategies such as interactive digital channels (apps, social networks) and more practical approaches are developed. This also includes the selection of healthy and sustainable Mediterranean products, through collaboration with food companies, to be offered to families according to their tastes and preferences. These tools could be applied in different contexts (e.g., school and family).

A3) **Deployment and assessment**: sample populations under investigation are randomized into different groups to test the newly developed strategies during a selected period: an analysis of the usefulness of the tools used individually and their combination is planned. During this phase, various endpoints will be analysed, such as changes in eating habits, socio-economic background, and anthropometric measures.

A4) **Consolidation and dissemination**: the most effective strategies are consolidated to be applied on a large scale in support of national and international public health policies both for the prevention and reduction of obesity and chronic non-communicable diseases in young people and their families and for environmental preservation. This activity also includes sharing the results of the project, through social media, scientific publications, and poster communications, with the aim of raising awareness on the usefulness of a multidisciplinary approach to promote adherence to a healthy and sustainable diet.

A5) **Writing and Editing** of the PhD thesis.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
A1)	***Consumer science and co-creation***																																				
A2)	***Development of innovative educational strategies and other tools***																																				
A3)	***Deployment and assessment***																																				
A4)	***Consolidation and dissemination***																																				
A5)	***Writing and Editing of the PhD thesis***																																				

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Characterization and quantification of *Fusarium* emerging mycotoxin(s) to improve food safety and quality

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This PhD thesis research project is aimed at predicting the risk of mycotoxin production by food transmitted fungal species by exploring their genomes and at developing innovative non disruptive mycotoxin(s) quantification methods to identify and quantify mycotoxins.

Caratterizzazione e quantificazione di micotossine emergenti prodotte da Fusarium per migliorare qualità e sicurezza dei prodotti alimentari

Questo progetto di ricerca ha lo scopo di predire il rischio di produzione di micotossine da parte di specie fungine emergenti esplorando il loro genoma e di sviluppare metodi innovativi e non distruttivi per la quantificazione di micotossine emergenti

1. State-of-the-Art

The increasing world population requires safe food and increased productivity, but the worldwide contamination of cereals and cereal products by mycotoxins, secondary metabolites produced by filamentous fungi, is of potential concern for human and animal health and food safety. Fusarium toxins, produced by various *Fusarium spp.*, are most frequently present as mixtures (Rodriguez and Naher, 2012). They include the well-known trichothecenes, fumonisins and zearalenone, but also the enniatins (ENNs), beauvericin, moniliformin and fusaproliferin, which are frequently referred as emerging mycotoxins (Jestoi, 2018). Emerging mycotoxins that are frequently present in cereals, especially wheat, barley, rye, and oats. These emerging mycotoxins have been poorly studied, despite their potential concern for human health. The presence of emerging mycotoxins in a wide range of cereal grains and their by-products, along with their co-contamination with other mycotoxins, has raised concerns about the lack of regulatory limits for these compounds. The European Food Safety Authority (EFSA) recognizes the potential chronic exposure risks but has not conducted a risk assessment due to the limited toxicity data available (Oueslati et al., 2011). Currently, the quantification of mycotoxins, relies on high-performance liquid chromatography-mass spectrometry (HPLC-MS). Although HPLC-MS is precise, it has drawbacks such as the use of chemicals, time consumption, high instrument costs, and sample disruption. Therefore, the development of alternative methods for mycotoxin identification and quantification is essential for improved monitoring in the food chain and for preventive risk assessment.

Exploring emerging mycotoxin risk can start from the ability of the strains to produce specific secondary metabolites. To estimate the potential risk, genomic information can guide the search for secondary metabolites likely produced by emerging threats in the food chain. For this purpose, the exploration of the genomes of *F. musae* and the investigation of secondary metabolite gene clusters with bioinformatic approaches will be investigated to assess the potentiality to produce emerging mycotoxin by this emerging food and health threatening species (Valenti et al., 2022).

Aptamers, which are short single-stranded DNA or RNA molecules, offer a promising alternative to conventional quantification methods. Aptamers can bind to target ligands with high affinity, similar to antibodies, and their specificity can be exploited in array-based diagnostics. Aptamers are synthesized through an in vitro evolution procedure called SELEX (Systematic Evolution of Ligands by EXponential Enrichment). This iterative selection process generates aptamers with the desired function, sensitivity, and selectivity. Aptamer-based quantification can be performed using a simple qPCR machine, making it accessible to most research laboratories (Rowe et al., 2009).

Near-infrared spectroscopy (NIR) is another alternative method for mycotoxin detection in food. NIR offers a simple, fast, non-destructive, and chemical-free approach, making it environmentally friendly. It provides information about molecular vibrations in the tested object and has been used for quantifying some mycotoxins. Hyperspectral imaging (HSI), a novel approach that improves upon conventional NIR devices, provides spectral information from each pixel of the captured image. It is particularly useful for analysing heterogeneous samples. Diffuse reflectance and push broom imaging are the preferred techniques for HSI measurement in grain evaluation (Orina et al., 2017; Fox and Manley, 2014).

In this scenario, the project aims to develop and validate novel methods for mycotoxin quantification and to assess genomic information from emerging Fusarium species to assess the toxigenic potential of the species.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

WP1: **Genome analysis:** (1.1) Entire genome of Fusarium pathogens will be sequenced following standard and assembled (1.2) combining short and long reads technology and using software such as Galaxy and Geneious, Functional Analysis (1.3) on obtained assembly will add gene function after annotation, identifying secondary metabolite potential production.

WP2: **Sampling:** Greenhouse experiment (2.1) conduced on Wild Type and esyn1 KO transformed strains of *F. avenaceum* and field data (2.2) from different fields will produce the collection of wheat on which mycotoxins will be quantified using standard quantification methods (2.3) and will produce the reference for the two methods under development.

WP3: **Aptamer:** Selecton of Aptamer (3.1) using SELEX process will be the first step followed by the set up step (3.2) in which will be carried out sensitivity selectivity and robustness of the method and then tested (3.3) on the obtained collection in previous WP

WP4: **HIS-NIR:** Hardware setting (4.1) for continuous photo collection that will be used for the set up phase (4.2) in which will be carried out the sensitivity and selectivity, final test on the collection (4.3) will be the final step before validation.

Table 1 Gantt diagram for this PhD thesis project.

		F	M	A	M	G	L	A	S	O	N	D	G	F	M	A	M	G	L	A	S	O	N	D	G	F	M	A	M	G	L	A	S	O	N	D	G
1	**Genome analysis**																																				
1.1	Extraction and sequencing																																				
1.2	annotation																																				
1.3	functional analysis																																				
2	**Sampling**																																				
2.1	greenhouse data																																				
2.2	field data																																				
2.3	HPLC-MS quantification																																				
3	**Aptamer**																																				
3.1	Selection																																				
3.2	set up																																				
3.2	test																																				
4	**HIS-NIR**																																				
4.1	hardware setting and photo collection																																				
4.2	set up																																				
4.3	test																																				
	Thesis and papers																																				
	Papers																																				
	thesis																																				

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Study and characterization of novel ingredients recovered from plant material for application in novel foods, by advanced chromatographic technique and high-resolution mass spectrometry

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This PhD thesis research project is aimed to study and chemical characterize plant materials potentially rich in bioactive and/or flavoring compounds, in order to develop new ingredients for food industry.

Studio e caratterizzazione di ingredienti innovativi recuperati da materiale vegetale, da applicare in novel food, tramite tecniche cromatografiche avanzate e spettrometria di massa ad alta risoluzione

Il presente progetto di tesi di dottorato sì pone l'obiettivo di studiare e caratterizzare chimicamente substrati vegetali potenzialmente ricchi in molecole bioattive o composti aromatici, con lo scopo di sviluppare dei nuovi ingredienti utilizzabili in diversi settori dell'industria alimentare.

1. State-of-the-Art

In the last few years natural products, derived from several types of plant materials, are attracting the attention of an increasing number of companies interested in the application of plant-derived additives in the food sector (Essien *et al*., 2020). The demand for the application of bioactive food ingredients derived from plant materials has increased in recent years. Natural compounds derived from plants have the potential to be new sources of food components and environmentally beneficial food preservatives.

***Table** 1 Some plant materials or biomasses as source of bioactive and aroma compounds.*

Plant source	Aim	Compounds/ aroma	Results	Application	Reference
Hemp microgreens/by-products	Chemical characterization of different varieties of hemp cultivated as microgreens.	Organic acids, amino acids, polyphenols and non-psychoactive phyto-cannabinoids	High amount of malic acid, citric acid, and tartaric acid; Highly content of total AA and EAAs in some cultivars; a good amount of phenolic acid and flavonoids; low level of psychoactive phyto-cannabinoid (Δ9-THC) and high concentration of cannaflavin A and B.	Potential use as an innovative functional food	Pannico et al., 2022
Brassicaceae microgreens	Chemical analysis of Brassicaceae microgreens to understand the impact of crop factors involved in the production of phytochemicals of different species.	Phenolic compounds, vitamins (A, D, E, K) glucosinolates, anthocyanins, isothiocyanates and indoles	High amounts of bioactive compounds when the fiber is used as substrates; artificial lighting could increase the phytochemical accumulation; bioactive compound profiles could change in response to fertilization.	Potential use in the food industry as a source of bioactive compounds	Alloggia et al., 2023
Kiwi fruit by-products	Chemical evaluation of new natural source of flavor and aroma metabolites	Volatiles organic compounds (phenyletyl alcohols, aldeydes, acids, chetons, furan)	The use of cheap substrates like kiwi fruit peel could be an interesting way to obtain 1-octen-3-ol (mushroom aroma), β-pinene (herbal, pine aroma), 3-octanol (mushroom, herbal aroma).	Natural flavors and aromas through low-cost substrates	Lindsay et al., 2022

Plant materials may represent excellent sources of nutrients and phytochemicals and for this reason, the potential use of them was evaluated as a more sustainable substitute of synthetic molecules (i.e., preservatives, antioxidant agents and flavoring). The use of plant materials or valorization of plant waste may play an important role in environmental sustainability for developing new ingredient products. Many plants material, as reported in Table

1, have been used to extract bioactive molecules or flavoring for a food application. In the past few years, microgreens have become more popular as new food with high concentrations of bioactive compounds, and it has been shown that modulation of growing conditions improves the phytochemical profile (Pannico *et al.,* 2022; Alloggia *et al.,* 2023). Likewise, also fruit by-products such as kiwi peel may be an interesting source of volatile organic compounds (Lindsay *et al.,* 2022). Currently, the widest extraction method used in the plant industry are not compatible with food industry. For these reasons, green technologies (i.e., pulsed electric field (PEF)) or supercritical fluid extraction are becoming increasingly preferred extraction methods to recover bioactive molecules in order to reduce the use of organic solvents. Thus, this PhD thesis will be aimed to select a plant material that can undergo extraction of bioactive compounds and subsequent chemical characterization, as to evaluate the re-use of the characterized by-product as potential ingredient in food preparations.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) **Preliminary assessment of plant materials** both in vitro culture and plant waste.
A2) **Extraction of bioactive compounds and VOCs** through conventional and non-conventional techniques.
A3) **Preliminary assessment of bioactive compounds** applying several assays and evaluate the antimicrobial and cytotoxicity activity of more promising extracts. The most promising extracts will be used to fortify food products.
A4) **Advanced chemical characterization of bioactive compounds and VOCs**
A5) **Bio-accessibility of Phenolic Compounds**
A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram of PhD activity during next two years.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Preliminary assessment***																								
	1) *In-vitro* plant growth																								
	2) Recovery of plant or fruit material by-product																								
A2)	***Extraction of compounds***																								
	1) Conventional extraction techniques																								
	2) Green techniques																								
A3)	***Preliminary assessment by assays***																								
	1) Polyphenol content																								
	2) Antioxidant activity																								
	3) Antimicrobial activity																								
	4) Cytotoxicity assay																								
	5) Testing on food products																								
A4)	***Advanced chemical characterization***																								
	1) UHPLC-MS/MS																								
	2) HS-SPME/GC-MS																								
A5)	***Bioaccessibility***																								
	1) *In-vitro* digestion																								
	2) Transepithelial trasposrt in Caco-2 cell																								
A6)	***Thesis and Paper Preparation***																								

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Engineering of microorganisms for the production of metabolites of interest and the transformation of molecules during fermentation processes

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The aim of this PhD project is to study and optimize lactic acid bacteria (LAB) fermentation processes capable to metabolize substrates to produce or degrade molecules of interest for the food sector. At first, the ability to degrade molecules, with focus on allergens, will be investigated. At the beginning, the research will be aimed to explore the diversity between the strains, investigating their proteolytic activity (*in silico*) and optimizing the fermentation parameters (*in vitro*). Analyzes will follow to verify its effect on allergens. The last phase will be directed to engineering the most promising LAB for optimizing the degradation process.

Ingegnerizzazione di microrganismi per la produzione di metaboliti di interesse e la trasformazione di molecole durante processi di fermentazione

Lo scopo di questo progetto di dottorato è quello di studiare e ottimizzare processi di fermentazione con batteri lattici (LAB) in grado di metabolizzare substrati per produrre o degradare molecole di interesse per il settore alimentare. Inizialmente, verrà valutata la capacità di degradare molecole proteiche, con focus sugli allergeni. La prima parte della ricerca sarà pertanto rivolta ad esplorare la diversità tra i ceppi, studiandone l'attività proteolitica (*in silico*) e ottimizzando i parametri di fermentazione (*in vitro*). Seguiranno delle analisi per verificarne l'effetto sugli allergeni. L'ultima fase sarà indirizzata all'ingegnerizzazione dei LAB più promettenti per ottimizzare il processo di degradazione.

1. State-of-the-Art

Climate change and population growth are driving the search for more sustainable feed and alternative foods to conventional sources. In this context, new protein sources could be introduced into the food chain, raising many health problems, especially in the field of allergies. Overall, more than 170 foods can cause allergic reactions in humans, but 90% of these allergies are induced by allergens from 8 major foods, including shellfish, soy, peanut, milk, tree nut, egg, wheat and fish. Food allergy corresponds to a type I hypersensitivity (mediated by immunoglobulin E, IgE) to otherwise harmless food proteins of animal or plant origin (allergens), affecting 5-10% of children and 1- 5% of adults. These allergens bind to IgE, on the surface of basophils or mast cells, and cause the release of pro-inflammatory mediators, such as histamine, and induce the symptomatic phase of allergy, causing urticaria, rhinitis, swelling, anaphylactic shock, death, etc. (El Mecherfi *et al.*, 2020; Pi *et al.*, 2021). Therefore, a significant challenge for global food safety and health concerns the study of new strategies to reduce food allergenicity.

Among the various strategies being studied at the moment, fermentation with LAB, in addition to improving the physical-chemical properties and nutritional values of foods, can represent an excellent solution as it allows to modify the protein structure and consequently reduce the sensitivity of the human body to food allergens. In fact, LAB play an essential role in food fermentation processes. LAB are auxotrophic and depend on their proteolytic system to meet their nutritional needs for amino acids in food matrices deficient in nitrogen sources necessary for growth. (El Mecherfi *et al.*, 2020). Thanks to this proteolytic system composed of cell envelope proteinases (CEP) the LAB can deactivate IgE epitopes by degrading the proteins into oligopeptides which are subsequently taken up by the cells via specific peptide transport systems for further degradation into oligopeptides and amino acids by means of a concerted action of various intracellular peptidases (Guo *et al.*, 2016; Pescuma *et al.*, 2015). Through a comparative genomic analysis, it was observed that the number of CEP genes can vary from one to four in a specific strain and that the simultaneous presence of two or more CEPs can improve the efficiency of breakdown of the proteins of interest (Guo *et al.*, 2016).

In this context, this PhD project will be aimed at investigating the proteolytic activity of LAB strains, selected among those belonging to the University of Parma Culture Collection (UPCC), aimed at the degradation of allergens. The characteristics of the different strains and allergens of interest will then be explored, verifying the presence of CEP proteases and applying different growth (time, temperature, inoculum concentration, etc.) in the presence of the allergens. We want to conduct a study not only on different types of LAB strains, but also on allergens from different food proteins, in particular insects, soy and gluten.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) ***In silico* analysis of LAB genomes to study the distribution of CEP proteases.**
A2) **Test the proteolytic activity *in vitro*** by fermentation processes applying different growth conditions (time, temperature, inoculum concentration, etc.) in the presence of allergens, by using LAB extracts or enzymes purified from different LAB strains.
A3) **Identify the metabolites produced during fermentation and verify the degradation of the allergens** using different techniques.
A4) **Optimization of the process through the engineering of the strains.**
A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***In silico* analysis**																								
A2)	**Test the proteolytic activity *in vitro***																								
A3)	**Identification of metabolites**																								
A4)	**Strain engineering**																								
A5)	**Thesis and Paper Preparation**																								

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Innovative food manufacturing by robotic technology

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The aim of this PhD thesis research project is to extend the application of the Industry 4.0 in the food sector through the application of robotic technology. The activities will firstly focus on one of the main unit operations, kneading/mixing, to explore possible benefits of robotics in this context. In addition, the research activities will be dedicated to the integration of robotics into the 3D printing technology, attempting to maximize the benefits offered by these industrial innovations impacting on the process efficiency and efficacy and on the quality of the end-products.

Processi alimentari innovativi attraverso la tecnologia robotica

Questo progetto di tesi di dottorato è dedicato allo studio ed estensione delle applicazioni dell'Industria 4.0 nel settore alimentare attraverso l'impiego della robotica in alcune fasi dei processi di produzione degli alimenti. Le attività si focalizzeranno su una delle principali operazioni unitarie, la miscelazione/impastamento, per esplorare i potenziali miglioramenti qualitativi conferiti dalla robotica in questa fase di processo. Inoltre, le attività di ricerca studieranno la possibilità di integrare la robotica con la tecnologia di stampa 3D per impiegare i maggiori gradi di libertà garantiti dalla robotica per la creazione di prodotti alimentari innovativi per forma, dimensione, proprietà interne e funzionalità chimico-fisiche.

1. State-of-the-Art

During the last years, several events such as the pandemic, the climate change and the recent Russia-Ukrainian war have caused economic, societal and environmental changes. Due to these events, we are witnessing an exceptional evolution of all those sectors that fall within the global economic sector. An extraordinary innovative approach that is fuelling the interest of academia and industries is the application of precision manufacturing. Such approach can be defined as the creation of a single objects by tailoring the process to the specific properties of both the raw materials and desired functionalities. These objects are produced with a precision impracticable by the traditional manufacturing processes. The robotic technology is one of the most important candidates to make reliable the ambitions of the precision manufacturing in the food sector because it is very flexible and with an extreme adaptability to the high variance of food properties. Many benefits could be reached by robots since they ensure more efficient processes, improved health, safety, security, reduced waste and environmental effects as well as mitigating labour-intensive tasks, as reported in Table 1 (EU, 2021). Despite these opportunities, while in different sector (i.e., the automotive and electronic sector) robots are already integrated in the production system, it is not realized and implemented with only few applications such as considering food handling, food packaging or the phase of cutting/peeling of food (Wang et al., 2021; Kanegae et al., 2020; Mu et al., 2019). However, there are still many other challenges to be faced for the practical and wide implementation of the robotic in foods industry such as the needs of detailed information regarding the main physical, mechanical and morphological features of food to be submitted to robotic food processing. Also, a large number of unit operations requiring for high level of standardization and precision as well as high level of hygiene have not been investigated in detail.

For instance, during the mixing/kneading operation of liquid-to-liquid, liquid-to-solid compounds and dry ingredients, different issues are faced due to the large variety of chemical and physical properties of materials. Many food applications require high spatial homogeneity of ingredients and additives, and a precise temperature control during heating and cooling processes to avoid segregated portion of the mixture. These questions combined with the high energy consumption make the mixing industry one of the less efficient (Ye & Chau, 2007). The use of robotic unconstrained movements during mixing could enhance the efficacy and efficiency of this operation.

Another potential interesting application of robots is in the field of Additive Manufacturing of food which is subjected to the growing interest of academia and food industries due to its ambitions of personalized manufacturing, on-demand production, high flexibility, etc. The introduction of robotics in 3D food printing would allow to obtain unparallel improvements due to the hundreds of degrees of freedom at disposal for unconstrained movements and precision dosing of food materials (Prashar et al., 2022). In fact, robotic movements open to the possibility to precisely control food composition and nutritional content creating customized foods based on individual dietary needs.

Table 1 - Potential benefits and capabilities of robotic technology in food manufacturing (from EU, 2021)

Industrial robot capabilities		Benefits
DECREASE	PRODUCTION COST MATERIAL WASTE	Reduced costs associated to manual labour and utility expenses; Increased efficiency for reduction of material waste and less scrap from rejects;
	FLOOR SPACE	Compact systems with mounting versatility;
	PRODUCTION TIME	Higher speed and efficiency, fast re-configurability;
IMPROVE	PRODUCT QUALITY	More efficient process control, high repeatability, and accurate task execution;
	PRODUCT UNIFORMITY	Errors caused by human error and fatigue eliminated;
	WORKING ENVIRONMENT	Existing labour upgraded, removes human from unfavourable conditions and tedious tasks;
INCREASE	PRODUCTION RATES	Ability to produce 24/7 without disruptions;
	FLEXIBILITY	Reconfigurable and easy to apply to a variety of tasks;
	SAFETY COMPLIANCE	Works in hazardous environments, made of hygienic materials;
	COMPETITIVE ADVANTAGE	Faster response to market demands, allows for product customization and personalization;
	EFFICIENCY	Optimized processes, increased yield (reduces production material waste, scrap from rejects).

2. PhD Thesis Objectives and Milestones

This PhD project aims to explore the integration of robotics into the food system improving the efficacy and efficiency of the food manufacturing sector. Specifically, the research activities will focus on two possible applications, mixing/kneading and additive manufacturing technology, according to the Specific Objectives (SO) listed in the Gantt diagram given in Table 2:

SO1. Develop, test and validate of a robotic kneading system with spatula or kneading hooks as end-effector: T1.1. will be focused on mixing test with model system and T1.2 with complex food formulation by modulating the main robotic variables (velocity, acceleration, temperature, time, mixing paths in the 3D space) and studying the effect of the main techno-functional properties of the samples.

SO2. Robotic 3D printing: during T2.1 some common and new variables of the 3D robotic arms will be modulated and their effect on several attributes (rheological, nutritional, physical, etc.) of the end-products will be analyzed. Research activity T2.2 will be dedicated to the development of uncommon geometries with more complex food formula.

SO3. Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 – Gantt diagram

Activity / Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
SO1) ***Robotic Mixing/Kneading***																								
T1.1 Kneading: model systems																								
T1.2 Kneading: complex formulation																								
SO2) ***Robotic 3D Printi ng***																								
T2.1 Process study and optimization																								
T2.2 Product development																								
SO3) ***Thesis and Paper Preparation***																								

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Valorization of agrifood residues for the development of highly active and environmentally friendly biofungicides

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The PhD research project aims at the development of novel and highly active antifungal agents to be used as biofungicides for crop protection, overcoming the emergency of resistant strains and limiting the use of harmful chemicals. A particular emphasis will be placed on the valorization of value-added bioactives present in agrifood residues by mild and efficient technologies. Scaffold optimization strategies, to increase both the activity and the bioavailability of the natural compounds, will be exploited taking advantage of green chemoenzymatic approaches.

Valorizzazione di residui della filiera agroalimentare per lo sviluppo di biofungicidi ad elevata attività e rispettosi dell'ambiente

Il progetto di ricerca mira allo sviluppo di nuovi antifungini ad elevata attività da utilizzare come biofungicidi per la protezione delle colture, per contrastare l'emergenza causata dall'insorgenza di ceppi resistenti e limitare l'uso di sostanze chimiche dannose. Particolare attenzione sarà posta al recupero e alla valorizzazione dei bioattivi presenti nei residui della filiera agroalimentare, mediante l'impiego di tecniche innovative. Partendo dalle molecole recuperate, al fine di aumentarne l'attività e la biodisponibilità, si procederà a cicli di ottimizzazione strutturale, anche mediante approcci chemoenzimatici.

1. State-of-the-Art

Fungi are among the greatest biotic threats to agricultural and food security. Fungal diseases cause between 10% and 23% of crop losses every year, plus a 10-20% of post-harvest leakage. All the five most important crops in the world (rice, wheat, maize, soya beans and potatoes) are affected by fungal infections. The pressure that a growing human population and global warming have on the food system, and the devastating impact fungi have and will keep having on the world's food supply, represent unprecedented challenges to food production. Fungi are highly infective and persistent, also because of the production of spores, and show an impressive plasticity and genetic variability. The increasingly widespread use of antifungal treatments, in addition to intensive monoculture cropping, provide an ideal environment for the emergence in fungal pathogens of fungicide-resistant strains. The current situation urges the discovery and development of novel, highly effective antifungal compounds (Stukenbrock *et al*., 2023).

In the past decade, the agrochemical industry has refocused its priorities moving towards the use of biological control agents (BCAs) including naturally occurring substances. The use of natural products from plant extracts as biofungicides has received increasing attention because of their high diversity and versatility. Especially in the context of sustainable agricultural development, research on the transformation of agrifood waste into high-value-added extracts or molecules has been intensified. Recently, it has been demonstrated that the production of numerous natural compounds by plants can be favored by adverse conditions, e.g. fungal infections. These secondary metabolites are usually acids or amides, enzymatically obtained as a self-defense mechanism. Under the stress conditions created during the infections, and because of the higher level of free reactive oxygen species, an increased formation of radicals is observed. Being highly unstable, radicals can react with other molecules and recombine their structure, resulting in the formation of new products such as dimers and trimers. These derivatives, having higher molecular weights, different spatial orientation and three-dimensional structure, are often responsible for a more specific and effective interaction with their biological target (Morimoto *et al*., 2018).

Cinnamic acids (ferulic, sinapic, caffeic, *p*-coumaric) are commonly found in plant cell walls of forage plants, or in cereals, vegetables, and fruits. They could be extracted as free acids, esterified with arabinoxylans and pectin, or as cinnamoylamides. Moreover, they could be recovered from agrifood waste and residues to be employed as such or as starting materials for the preparation of more complex derivatives. Cinnamic acids are often involved in dimerization reactions, due to the formation of reactive radical species. Ferulic acid and its derivatives, including dimers, have been recently reported as a promising class of antifungal agents. In particular, the poacic acid dimer is known to directly bind β-1,3-glucan, an important component of the cell wall, acting on the formation of glucan fibrils, and causing cell leakage. This mode of action is distinct from that of other antifungal agents targeting the cell wall, such as echinocandins. Furthermore, *in vivo* studies showed a significant decrease in the β-1,3-glucan synthesis after treatment with poacic acid. Other ferulic acid derivatives, belonging to the class of Hordatines (dimers of hydroxycinnamic acid amides) showed *in vivo* inhibition of the spore germination in early growth stages

of several fungal pathogens. Little is known about further functionalization of these compounds, or about their use in combination with other antifungals (Piotrowskia *et al*., 2015; Kohyama *et al*., 2013; Jia *et al*., 2018; Djande *et al.*, 2022).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Extraction, purification, and characterization of cinnamic acids** from agrifood waste.

A2) **Synthesis of a first collection of dimers from various cinnamic acids** by green chemistry approaches and biocatalyzed reactions.

A3) **Design and setup of a first series of biological test for the evaluation of the antifungal activity on various fungal strains (**in collaboration with plant pathologists). Tests will be an important part of my project since their result will lead to the identification of the most promising scaffolds and will guide the successive synthetic steps.

A4) **Identification of the lead compound and functionalization of the structure:** the result of the biological screening will allow to identify the most promising molecules for further investigation. Different functionalization will be introduced on the natural skeleton to obtain a larger collection of compounds hopefully endowed with high activity and/or bioavailability. In this part, it could also be considered the idea of developing molecules with a dual mode of action by functionalization of the lead structure with other antifungal moieties.

A5) **Optimization of the synthetic process and further investigation on the mechanism of action:** to obtain the most effective synthetic pathway and to better understand the molecular target and mechanism of action of the selected compounds.

A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 *Gantt diagram for this PhD thesis project*.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Extraction, purification and characterization of cinnamic acids***																								
A2)	***Synthesis of a first collection of dimers from various cinnamic acids***																								
A3)	***Design and setup of a first series of biological test for the evaluation of the antifungal activity on various fungal strains***																								
A4)	***Identification of the lead compound and functionalization of the structure***																								
A5)	***Optimization of the synthetic process and further investigation on the mechanism of action***																								
A6)	***Writing and Editing***																								

*If the synthetized molecules will show a low or no activity after the biological screening (A3), a new series of natural compound, known in literature to be active as fungicides, will be taken into consideration.

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Application of innovative technologies for the functionalisation of alternative proteins and the associated functional and rheological characterisation

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The growing market for plant-based products necessitates specific ingredients to address new technological challenges. Non-thermal technologies, including cold plasma (CP), pulsed electric field (PEF), ultrasound (US), and high-pressure processing (HPP), offer sustainable and efficient approaches for protein functionalization. This PhD project aims to evaluate the use of these innovative treatments on legume flours, studying their effects on functional properties. The modified flours and their potential applications in bakery, snack, or dairy replacement products will be assessed. The project also investigates the environmental sustainability of the functionalization process. The research contributes to understanding protein modification techniques for developing improved plant-based alternatives.

Applicazione di tecnologie innovative per la funzionalizzazione di proteine alternative e relativa caratterizzazione funzionale e reologica

Il crescente mercato dei prodotti a base vegetale richiede ingredienti specifici per affrontare specifiche sfide tecnologiche. Le tecnologie non termiche, come CP, PEF, US, e HPP, offrono approcci alternativi per la funzionalizzazione delle proteine. Questo progetto mira a valutare l'utilizzo di tali trattamenti su farine di legumi, studiandone gli effetti sulle proprietà funzionali, per poi esplorare le potenziali applicazioni in prodotti da forno, snack o sostituti dei latticini. Il progetto si prefigge di analizzare anche la sostenibilità ambientale del processo di funzionalizzazione. Questo progetto mira a contribuire alla comprensione delle tecniche di modifica applicabili su farine proteiche.

1. State-of-the-Art

Following recent trends in the food sector, an increasing market share of plant-based products can be observed, ranging from dairy substitutes to alternative meats (GFI Europe, 2023), trend that is expected to increase further, also thanks to a shift in consumer preferences towards more environmentally friendly and cruelty-free products. Finally, especially in the richest countries, the development of protein foods from vegetable matrices has experienced tremendous growth (Fasolin *et al*., 2019; Aschemann-Witzel *et al*., 2021). As a result of this situation, more and more companies are launching such products, creating a need for specific ingredients. As it is well known, many plant-based products are characterised by a long list of ingredients used to overcome technological problems and meet specific requirements in terms of sensory properties and stability, to mimic the animal counterpart (Akharume *et al*., 2021). Unfortunately, proteins derived from plant sources differ not only in terms of nutritional value but also in terms of technological properties. Solubility and gelling properties are generally lower compared to those of animal origin (especially at pH close to neutrality), making their use in formulations more complex (Akharume *et al*., 2021).

One response to these needs can be the modification of plant proteins to obtain products with specific properties. Protein functionalisation has been used in the food sector for several years (Panyam and Kilara, 1996; Messens *et al.,* 1997). Recently, however, the interest of scientific research has shifted from the traditional chemical-enzymatic modifications (e.g. glycosylation, acetylation, hydrolysis and cross-linking) to physical modifications obtained by applying non-thermal technologies such as cold plasma (CP), pulsed electric field (PEF), ultrasound (US), high-pressure processing (HPP) and extrusion, which make the whole functionalisation process more sustainable and efficient (Mirmoghtadaie *et al*., 2016; Sun-Waterhouse *et al*., 2017; O'sullivan *et al*., 2022).

Although the four technologies mentioned above are all considered non-thermal, they are based on different functional mechanisms. CP is able to favour the rearrangement of the protein structure thanks to the main action of the reactive oxygen and nitrogen species (RONS) formed (Basak and Annapure, 2022), while in HPP, the denaturing effect on the proteins is achieved by the compression that causes the collapse of the structures with empty spaces (such as the β-sheets) (Wang *et al*., 2022). One of the most studied technologies is the application of US, where the short and localised pressure and temperature shocks (thanks to the cavitation phenomenon) can act both at the macroscopic level on the size of the particles and at the microscopic level, denaturing the proteins and exposing the most lipophilic areas (O'sullivan *et al*., 2022). Finally, the least used technology for this purpose is PEF, as its efficacy and mechanism of action on proteins is still controversial (Han *et al*., 2018).

2. PhD Thesis Objectives and Milestones

The aim of the project is to evaluate the possibility of using innovative non-thermal treatments to modify the functional properties of legume flours and subsequently use these optimised ingredients in product formulation to meet specific needs. The project can be divided into the following tasks, which are also time-framed in Table 1.

A1) Literature review of previous studies on the application of non-thermal technologies for flour modification and protein denaturation and research on the specific needs in the formulation of new plant-based products.
A2) Evaluation of the properties of different legume flours with the aim of identifying one or two specific legumes to work on.
A3) Application of different treatments to the selected flours, with the aim of studying the effects on functional properties. The effect of PEF, US, HPP and CP will be evaluated and optimisation of parameters for the best treatment will also be studied.
A4) Evaluation of the functional and rheological properties of the modified flours, with the aim of identifying some key aspects in which the flour has been modified and try to find a suitable use in a final product.
A5) Development of a bakery, snack or dairy substitute product using the ingredients obtained to understand if the functionalisation process can improve the performances of the final product.
A6) Assessment of the sustainability level of the overall functionalisation process. An environmental and economic assessment will be done for each newly developed ingredient, taking into account other, more conventional functionalisation systems.
A7) Writing and editing of the final dissertation, scientific papers and attending conferences with oral and/or poster presentations.

Table 1: Gant chart with the expected duration of different research activities.

Activity / Months		2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1	Literature review and research	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
A2	Flour characterization and identification of target characteristics	■	■	■	■														
A3	Treatments application and optimization for flour functionalization (PEF, US, HPP, CP)			■	■	■	■	■	■	■									
A4	Quality evaluation and identification of possible usages for the product						■	■	■	■	■	■	■						
A5	Application of the created flours for new final products										■	■	■	■	■				
A6	LCA and LCC of developed processes												■	■	■	■	■		
A7	Writing dissertation, scientific papers	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

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Study on the modulation of the intestinal microbiota induced by nutraceutical preparations designed to improve immune and metabolic function

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This project aims to explore the effects of plant extracts (PEs) used as dietary supplements, gut microbiota and gastrointestinal health. The objective is to determine if these not only alleviate symptoms but also improve overall health and prevent other conditions without adverse effects on gut microbiota. The project begins with *in vitro* testing using colonic fermentations to evaluate PEs' effects on gut microbiota populations and growth tests on single strains will assess their impact on health-promoting bacteria. The most promising PE will be selected for a human study to investigate its prebiotic effect and influence on health biomarkers.

Studio sulla modulazione del microbiota intestinale indotta da preparazioni nutraceutiche progettate per migliorare la funzione immunitaria e metabolica

Questo progetto mira a esplorare gli effetti di estratti vegetali (PE), usati come integratori alimentari, sul microbiota intestinale, sulla salute gastrointestinale e determinare se questi non solo alleviano i sintomi, ma possono migliorare lo stato di salute senza effetti negativi sul microbiota intestinale. Il progetto prevede test *in vitro* utilizzando fermentazioni coloniche per valutare gli effetti dei PE sulle popolazioni del microbiota intestinale e test di crescita per valutare l'impatto su singole specie batteriche intestinali note per la loro attività benefica. Il più promettente sarà selezionato per un feeding trial per indagare effetto prebiotico e influenza su biomarker della salute.

1. State-of-the-Art

Plant extracts (PEs) have the ability to affect members of the gut microbiota. This impact can be either negative, targeting beneficial bacteria, or positive, by acting against harmful bacteria or pathogens. For instance, this can be attributed to the antimicrobial or prebiotic activity of certain compounds found in the extracts. While commercially-available prebiotics are typically carbohydrates, non-carbohydrate compounds such as polyphenols can also confer health benefits through their metabolization by intestinal microorganisms. Polyphenols have been observed to increase the levels of *Akkermansia* spp., bifidobacteria, and lactobacilli in mice. Flavonols derived from plant sources like cocoa and green tea have also exhibited prebiotic activity (Neri-Numa et al., 2020). Health can also be influenced by the production of microbial metabolites, which is impacted by food intake. For example, short-chain fatty acids (SCFAs) and trimethylamine N-oxide (TMAO). SCFAs play a critical role in gut health by providing energy to epithelial cells, supporting nutrient absorption, reducing gut inflammation, and maintaining the gut barrier. Elevated TMAO levels are associated with an increased risk of cardiovascular diseases, as it promotes inflammation, impairs cholesterol metabolism, and affects platelet function. Analysing the prebiotic effect and modulation of the microbiome *in vivo* poses significant challenges. This is primarily due to the unique nature of each individual's microbiota, making it difficult to make predictions at a population level. To overcome this, techniques have been developed to simulate an active microbiota. One common approach involves using a standardized pool of faecal samples as an inoculum, which is then subjected to anaerobic conditions to initiate "colonic fermentations." Another crucial aspect to consider is the viability of the microorganisms in the faecal inoculum (Liu et al., 2021). Using glycerol-supplemented faecal samples prepared under anaerobic conditions, helps maintain up to 95% viability of the microbial populations for six months (Smirnova, 2019). In the context of simulating the microbiota, the *in vitro* digestion protocol proposed by INFOGEST provides us with a standardized tool to simulate the impact on the human gut microbiota when applied to the food under investigation (Brodkorb et al., 2019). Furthermore, population shifts analyses have notably advanced in the last decade, particularly with the increasing effectiveness and accessibility of next-generation sequencing techniques. For instance, metataxonomics by 16S rRNA gene profiling can now provide taxonomic community structure in complex ecosystems and offer a detailed understanding of the prokaryotic composition at the family, genus, and even species levels, along with their respective abundances. Giving us new tools to exploit along the classical microbiology and molecular biology ones. While *in vitro* data holds value, conducting human feeding trials is

essential to acquire more reliable knowledge regarding the impact on human health. A human feeding trial on one PE will be an integral part of this project to ensure comprehensive understanding and an accurate evaluation.

2. PhD Thesis Objectives and Milestones

The main activities of the project are divided into the two work packages (WP) described below.

A) Screening of PEs: • Select the PEs for investigation. • Conduct *in vitro* digestion of PEs following the INFOGEST protocol. • Prepare a standardized faecal sample. • Perform "colonic fermentations" using digested and non-digested PEs. • Analyse changes in microbial populations using 16S rRNA gene profiling.
• Evaluate growth parameters of notable bacteria promoting host health in the presence of PEs.

B) *In vivo* human intervention trial: • Identify the most promising PE for the feeding trial. • Develop and present the trial protocol to the ethics committee. • Recruit healthy individuals to participate. • Conduct a placebo-controlled, randomized, double-blind, cross-over feeding study with the selected PE. • Collect faecal and urine samples at various timepoints. • Perform targeted and untargeted metabolomics analyses on the samples, focusing on specific targets relevant to the investigated plant extract (PE). • Analyse microbial population shifts in the subjects using 16S rRNA gene profiling.

This study aims to thoroughly examine the impact of PEs on gut microbiota and the resulting metabolite production. It will significantly contribute to our understanding of how PEs affect gut health and their broader implications for overall well-being. The collected data will help assess the safety of these products concerning the microbiome and gut health, potentially revealing new applications based on observed effects.

Table 1. *Gantt diagram of the project*

activity	months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
wp) A	literature review	■	■	■	■	■	■	■	■	■	■	■	■																								
	PE selection			■																																	
	INFOGEST				■	■	■																														
	colonic fermentation setup						■	■	■	■																											
	colonic fermentations								■	■	■	■	■																								
	gut strains growth with Pes				■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■											
wp) B	feeding trial preparation													■	■	■	■	■																			
	trial recruitment																		■	■																	
	feeding trial and sample collection																				■	■	■	■	■												
	microbiota analysis																								■	■	■	■									
	metabolomics																										■	■	■								
	data analysis and writing																													■	■	■	■	■	■	■	■

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Development of a "foodomics" platform for monitoring the transformation process of aromatic plants for the food industry

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Herbal plants find large use in the food industry for providing aroma to semi processed and processed food products. The aroma quality and quantity in the ingredient is function of multiple factors including the time of harvesting, the age of the plant and the condition of transformation. The present project aims at developing a multi-analytical model for characterizing at molecular level of herbal plants, using basil's leaves as the model, at different steps from farm to consumer. The multi-omic approach will help identifying qualitative markers for maximizing the aromatic traits of herbal plants.

Sviluppo di una piattaforma "foodomics" per il monitoraggio del processo di trasformazione alimentare delle erbe aromatiche

Le piante erbacee trovano ampio impiego nell'industria alimentare per fornire aroma a prodotti alimentari semilavorati e lavorati. La qualità e la quantità dell'aroma nell'ingrediente è funzione di molteplici fattori, tra cui il momento della raccolta, l'età della pianta e lo stato di trasformazione. Il presente progetto mira a sviluppare un modello multi-analitico per la caratterizzazione a livello molecolare di piante erbacee, utilizzando le foglie di basilico come modello, in diverse fasi dall'azienda agricola al consumatore. L'approccio multi-omico aiuterà a identificare marcatori qualitativi per massimizzare i tratti aromatici delle piante erbacee.

1. State-of-the-Art

Aromatic plants, known also as "herbs and spices", find large application in the food industry as a source of uniquearoma and taste and in the production of functional foods, taking advantage of their bioactive compounds (bactericidal, anti-oxidative, fungicidal).
Plants belonging to the Lamiaceae family are particularly intriguing because of their natural ability to produce and accumulate "essential oils." Basil (*Ocimum basilicum* L.) plays a crucial role in Mediterranean cuisine because ofits distinctive aromas. It is also considered a notable herb, with researchers studying its genotypic characteristics and the effects of consecutive harvests on the phenolic acids and aromatic profile (Ciriello et al., 2021). Basil leaves are widely used in preparing pesto and as a seasoning for fresh or semi-prepared dishes. The basil essential oils are synthesized in specialized leaf epidermal outgrowths called glandular trichomes and include predominantly terpenes such as oxygenated monoterpenes, hydrocarbon sesquiterpenes, oxygenated sesquiterpenes, and phenylpropanoids. These compounds are well-known for their antioxidant properties, providing additional benefits beyond their aromatic qualities. The content of bioactive phytochemicals in basil depends also on various factors, including the variety, growing conditions, altitude, storage, location, and weather. Subsequent harvests can influence the qualitative characteristics, playing a pivotal role in defining the final sensory profile of the product. As regard basil, Terpenes (monoterpene: (*R*)- linalool and 1,8-cineole and sesquiterpene: germacrene D and α-bergamotene being sesquiterpenes) and phenylpropenes are the primary components of basil's essential oils. The growing interest for medicinal properties as well as for economic im- portance in Lamiaceae species has led to the publication of several genomes in recent years (Vining et al., 2022; Bornowski et al., 2020; Hamilton et al., 2020; Jia et al., 2021; Zheng et al., 2021; Li et al., 2022), thus providing an opportunity to exploited available genomic resources for the assessment of food product quality and safety. Three basil genome assemblies were released (Table 1), the sweet basil variety 'Perrie' will be used in this study to develop a novel and advanced platform in food science and technologies.

Table 1. *List all the available genomic resources of the Ocimum basilicum cultivars*

Cultivar name	Genome size (Gb)	Predicted gene set	References
Perrie	2.13	-	Dudai *et al.,* 2018
Perrie	2.13	62,067	Gonda *et al.,* 2020
Genovese	2.068	78,990	Bornowski *et al.,* 2020

2. PhD Thesis Objectives and Milestones

The present project aims at creating a multi-analytical system to characterize aromatic plants and standardize and evaluate the process of transformation of their products. The approach will be based on genomic, transcriptomic, and proteomic data to assess how the genetic traits control and regulate the aromatic profile changes during the plant's development. This will be supported by metabolomic data to create "foodomic" markers to support the food business operators in choosing the best processing conditions. The multidimensional platform will have two main purposes: to create a signature for ingredient authentication and to monitor the evolution of molecular components during transformation processes. Additionally, the developed model can be applied to other plant varieties. The entire project will be carried out through an integrated and multidisciplinary exchange between different scientific areas.

The PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) **Development and optimization of sample preparation process** for each target analytical platform.

A2) **Generation of data** for each target platform.

A3) **Elaboration, interpretation, and visualization of multi-omic data** to understand the genetic traits thatcontrol and regulate the aromatic profiles change during the plant's development.

A4) **Manuscript preparation and submission of the scientific papers** and oral and/or poster communications.

A5) **Writing and Editing of the PhD thesis**.

Table 2. *Gantt diagram for Ph.D. thesis project.*

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Sample preparation process***																								
A2)	***Generation of Data***																								
A3)	***Analyses of multi-omic data***																								
A4)	***Preparation of the scientific papers***																								
A5)	***Thesis Preparation***																								

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Design and development of meat and dairy analogues using vegetable proteins from upcycled sources

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This PhD research project is aimed at valorising alternative proteins extracted from plant by-products towards their use in the design and development of meat and dairy analogues. This general objective will be pursued starting from the characterization and improvement of the protein's techno-functional properties, thus selecting the most promising ones for the optimization of formulations and processes to obtain safe, nutritionally valuable, and sensory acceptable products.

Progettazione e sviluppo di analoghi della carne e di prodotti lattiero-caseari mediante l'impiego di proteine estratte da sottoprodotti di origine vegetale

Questo progetto di dottorato ha come scopo quello di valorizzare proteine alternative estratte da sottoprodotti vegetali mediante il loro utilizzo nella progettazione e nello sviluppo di analoghi della carne e di prodotti lattiero-caseari. Questo obiettivo generale sarà perseguito a partire dalla caratterizzazione e miglioramento delle proprietà tecnico-funzionali delle proteine studiate, selezionando quelle più promettenti per l'ottimizzazione di formulazioni e processi volti ad ottenere prodotti sicuri, nutrizionalmente validi e sensorialmente accettabili.

1. State-of-the-Art

Over the past decade, the increase in the global population and the growing awareness towards the sustainability impact of livestock farming have generated a large demand for alternative eco-friendly proteins that can meet the nutritional needs of the population, without excessively impacting the ecosystem (Aiking and de Boer, 2020). To ensure a proper integration into the human diet, proteins should be safe, nutritionally valuable, and should be characterized by adequate functional and sensory characteristics that allow their inclusion in food formulations. In this regard, plant-based proteins have emerged as promising candidates, and their use is already widespread. Soybean, pea, and gluten are the most used raw material due to their availability, affordability, and high functionality. However, many other proteins sources are potentially suitable for these kinds of applications and are currently object of investigation. In this context, considering that approximately 14 percent of the food is globally lost or wasted along the agri-food chain between harvest and retail market (FAO, 2019), and that high quantities of this material are rich of proteins, plant by-products represent an interesting source of alternative proteins (Kamal et al., 2021). Utilization of plant proteins is not always straightforward; some strategies have been studied over time to improve their sensory, nutritional, and technological properties, including fermentation and high hydrostatic pressure (HPP) (Akharume et al., 2021; Avelar et al., 2021).

The demand for meat and dairy analogues is growing rapidly because of the shift toward plant-based diets due to concerns about human health, animal welfare, and environment (He et al., 2020). Developing such products is challenging due to the different structural and functional characteristics of animal-derived ingredients compared to the plant-based ones.

Plant-based alternatives to dairy products can be produced starting from mixture of isolated ingredients (such as water, plant proteins, vegetable oils, starch/hydrocolloids, salts, and starter cultures) or from whole plant material that can be soaked and then destroyed to form a colloidal structure. In both cases the result is a colloidal dispersion that can be homogenized and structured through different methods and processes, including enzymatic reactions, fermentation, acid/salt addition, and heat-gelation (Grossmann and McClements, 2021).

Plant-based meat analogues can include imitations of products such as hamburgers, sausages, patties, chicken nuggets, steaks, and more. A major challenge in the development of this type of product relates to the commonly globular structure of plant-proteins, strongly different if compared to the elongated fibrous shape of meat proteins. Therefore, technologies have been developed for texturizing plant proteins to give them a fibrous shape (e.g., high, and low moisture extrusion, shear cell technology, and electrospinning) (Baune et al., 2022).

This PhD project is part of the IPSUS project "Climate-smart food innovation using plant and seaweed proteins from upcycled sources" (ERA-NETs SUSFOOD2 and FOSC Joint Call) which is aimed at using innovative approaches to explore opportunities for upcycling plant and seaweed proteins from agri-food raw materials of different origins (pumpkin, hazelnut, grape, potato, brewers' spent grain, seaweeds).

The purpose of this PhD project is primarily, to investigate the techno-functional properties (e.g.: gel-forming capacity, emulsifying capacity, foam-forming capacity, solubility, water absorption capacity, oil absorption capacity), of alternative and under-studied proteins extracted from the selected by-products, and to test

technological approaches aimed at their modification (HPP, Fermentation). The second objective will be to use the most promising alternative proteins for the formulation of alternative meat and dairy prototypes by testing different technologies and optimizing formulation and process parameters by a design of experiment approach.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Literature research.

A2) Characterization and modification of proteins techno-functional properties: Characterization of the selected proteins techno-functional properties (Foaming, Emulsifying, Gelation, Solubility, WAC, OAC) **(A2.1);** testing the effect of conventional and alternative technologies (e.g., Fermentation, HPP) **(A2.2).**

A3) Design and development of plant-based cheese prototypes: Selection of the most promising upcycled proteins (output of A2), formulation and optimization of the recipe, based on marketed products, scientific literature, and pre-testing **(A3.1);** Comparison and optimization of different technological unit operations for emulsifying (HPH) and structuring (Heat-treatment, acid/salt addition, fermentation) the ingredients **(A3.2);** Characterization of functional and sensory properties of the prototypes **(A3.3)**.

A4) Design and development of plant-based meat prototypes: Selection of the most promising upcycled proteins (output of A2), formulation and optimization of the recipe, based on marketed products, scientific literature, and pre-testing **(A4.1);** Optimization of process parameters for protein texturization (high moisture extrusion) **(A4.2);** Characterization of functional and sensory properties of the prototypes **(A4.3)**.

A5) Thesis and papers writing.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Literature research***																								
A2)	***Protein functionality***																								
	1) Characterization																								
	2) Modification																								
A3)	***Cheese alternatives development***																								
	1) Formulation design																								
	2) Process design																								
	3) Characterization																								
A4)	***Meat alternatives development***																								
	1) Formulation design																								
	2) Process design																								
	3) Characterization																								
A5)	***Thesis and papers preparation***																								

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Innovative management of maceration processes and stabilization techniques for the production of monovarietal wines

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This PhD project aims to find variety-specific tools aimed at improving the phenolic traits of monovarietal wines, with a particular focus on how phenolics are accumulated in the berry during ripening, extracted during maceration, and evolve over time. Several winemaking techniques will be tested to understand how they can affect the extraction and stabilization of phenolic compounds and to design novel approaches to use them to obtain variety-specific results.

Gestione innovativa dei processi di macerazione e delle tecniche di stabilizzazione per la produzione di vini monovarietali

Questo progetto di dottorato mira a trovare strumenti specifici per varietà al fine di migliorare i tratti fenolici dei vini monovarietali, con particolare attenzione alla cinetica con cui i composti fenolici si accumulano nell'acino durante la maturazione, vengono estratti durante la macerazione e si evolvono nel tempo. Verranno testate diverse tecniche di vinificazione per capire come possono influenzare l'estrazione e la stabilizzazione dei composti fenolici e per progettare nuovi approcci per utilizzarle al fine di ottenere risultati specifici per varietà.

1. State-of-the-Art

Italian grape varieties are famous for their wide diversity in terms of berry constituents. The variability among cultivars is important because it represents the differences that characterize the corresponding monovarietal wines (Giacosa et al., 2021). Therefore, monovarietal winemaking should be intended as a production process aimed at enhancing the characteristics of each variety. The quality of grapes is based on the content of primary and secondary metabolites that accumulate during ripening. In particular, the sugar and acid content and the total amount of extractable phenolic substances are important variety-dependent parameters, further influenced by the harvest date. Indeed, the accumulation kinetic of phenolic compounds in the berry differs according to the variety, the climatic conditions of the vintage, and the growing area (Cagnasso et al. 2011). Therefore, the evaluation of the accumulation kinetics of phenolic compounds in berry skins and seeds are crucial to plan the harvest date and to ensure the right extractable phenolic pool from grapes. The color characteristics and mouthfeel properties of red wines firstly depend on the extraction of phenolic compounds that takes place during grape skin maceration. To improve the extraction of phenolic compounds, several techniques are used. The use of enzymes has been proposed to increase the extraction of phenolic substances during the maceration. Their use facilitates the disruption of skin cell walls leading to an enhanced phenolic extraction. Enzyme preparations are marketed to increase phenolic extraction in a specific extent; however, the final result may be affected by the mix of primary and side enzyme activities featured in commercial mixture (Romero et al., 2008). Moreover, in literature contrasting results have been found according to the variety and the type of enzyme activity tested. Río Segade et al. (2015) obtained variable outcomes in terms of anthocyanin extraction by testing the same enzyme formulation on varieties with distinct anthocyanin profiles. On the other hand, various enzyme activities tested on the same variety led to a different phenolic extraction from grape skins, with consequently different implications on the wine chromatic characteristics (Bautista-Ortín et al., 2005). Tannins play an important role in color stabilization and in the determination of wine mouthfeel properties. Given that different maceration conditions (i.e., length, temperature) can affect the phenolic extraction, several techniques have been proposed in the literature to modulate the extraction of tannins from the seeds and skins during maceration, but different results have been obtained depending on the variety (Busse-Valverde et al., 2010). Few studies have been conducted on how to modulate the maceration conditions to achieve variety-specific results. Monomeric anthocyanins extracted from grape skins during the maceration can undergo various phenomena, such as reabsorption by cell wall materials, oxidation and interaction with other phenolic or non-phenolic compounds. These latter reactions result in the formation of more stable pigments. Copigments, anthocyanin-tannin and anthocyanin-anthocyanin adducts, and pyranoanthocyanins represent key compounds in the determination of wine colour and their formation is affected not only by the initial amount of phenolics in grapes, but also by the winemaking and storage conditions. In particular, the oxygen exposure drives the evolution of phenolic traits of wines after the alcoholic fermentation, as oxygen can react with di– and tri–hydroxyphenols to produce quinones and hydrogen peroxide, which can oxidize further substances. The effect of oxygen on the wine evolution has been deeply studied, nevertheless, high variability in the results was reported according to the wine composition (Petrozziello et al., 2018).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Deepening the understanding of varietal berry composition** by studying the kinetics of phenolic compound accumulation on selected varieties (A1.1) to find new solutions and tools (development of accumulation models, A1.2) to determine the optimal harvest date;

A2) **Evaluation of different maceration conditions** such as length, temperature, and cap management to identify how they can affect the phenolic extraction (A2.1) and how they can be modulated in varietal winemaking (A2.2);

A3) **Application of novel technological aids** to improve wine phenolic traits**,** with a particular focus on enhanced enzyme treatments applied during the maceration to increase the phenolic extraction (A3.1) and subsequent identification of possible specific applications for Piedmontese varieties (A3.2);

A4) **Study of the evolution of wine phenolic characteristics through aging** by exploring phenolic stabilization under different winemaking conditions (A4.1) and evaluating the chromatic characteristics of varietal wines after different oxygen exposure levels (A4.2);

A5) **Elaboration of tailor-made techniques for varietal enology**;

A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***In-depth study of berry composition***																								
	1) Evaluation of phenolic ripeness																								
	2) Models for phenolic accumulation																								
A2)	***Effect of maceration conditions***																								
	1) Influence of maceration techniques																								
	2) Variety-specific application																								
A3)	***Technological aids***																								
	1) Evaluation of enzymes performance																								
	2) Application in varietal enology																								
A4)	***Phenolics in wine evolution***																								
	1) Phenolic stabilization																								
	2) Effect of oxygen exposure																								
A5)	***Creation of variety-specific strategies***																								
A6)	***Thesis and Papers Preparation***																								

3. Selected References

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Recovery and valorization of exhausted fermentatioN brOths and by products to reduce WASTE in the agri food sector (NO WASTE)

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In agri-food industry, waste disposal of spent media from microbial biomasses production is a critical point. Thousands of liters of spent media are discarded, although still rich in nutrients. It implies a waste of nutrients and high environmental impact of their disposal. Among gut microorganisms, cross-feeding is known to take place with an exchange of metabolites between 'producers' and 'recipients' species. This project is aimed at recycling exhausted fermentation growth media from traditional probiotics biomass production to be used as source of purified fractions or additives to enrich industrial media needed for production of Next Generation Probiotics.

Recupero e valorizzazione di terreni di fermentazione esausti per ridurre gli scarti di produzione nel settore agro-alimentare

Nel settore agroalimentare, un punto critico della produzione industriale è lo smaltimento dei terreni esausti derivanti dalla produzione di biomasse. Migliaia di litri di terreno esausto vengono scartati, nonostante siano ancora ricchi di nutrienti. La conseguenza è uno spreco di nutrienti, il cui smaltimento ha un elevato impatto ambientale. Tra i microorganismi del microbiota intestinale avviene fenomeno di cross-feeding, cioè uno scambio di metaboliti tra specie differenti. Questo progetto è finalizzato al riciclo di terreni di fermentazione esausti, derivanti dalla produzione di probiotici tradizionali, per essere usati come fonte di frazioni purificate o additivi per l'arricchimento di terreni industriali utili per la produzione di probiotici di futura generazione.

1. State-of-the-Art

In the agri-food industry, the disposal of spent media resulting from the production of microbial biomasses is a critical point. After biomass preparation and separation, thousands of liters of spent media are discarded, even though still containing high level of sugar, protein, peptides, and other nutrients, as well as organic acid and other products of the primary and secondary metabolism of the cultured microorganism (Fenster *et al*., 2019). It implies a double issue: a waste of nutrients and the high environmental impact of their disposal. Trophic interactions can occur among microorganisms of the gut microbiome, implying an exchange of nutrients so that metabolites are released as product by a species (producer) and used as nutrients by another species (recipient). As an example, species that utilize a particular polysaccharide will liberate polysaccharide breakdown products that are consumed by other species unable to grow on the polysaccharide alone. Exchanges of short-chain fatty acids (SCFAs) (e.g., acetate, propionate, and succinate), organic acids (e.g., lactate), amino acids, and vitamins are common examples of metabolic interactions (Hirmas *et al*., 2022). An interesting example are the cross-feeding interactions occurring in the gut between *Bifidobacterium* and butyrate-producing bacteria, such as *Faecalibacterium prausnitzii*, *Roseburia*, and *Eubacterium* (Lee *et al*., 2018). The latter three genera belong to the Next-generation probiotics (NPGs), promising probiotics identified through comparative bioinformatic analyses in healthy subjects. They are more difficult to be cultivated compared to traditional probiotics, since they are nutritionally demanding and highly sensitive to aerobic conditions, which translates into several technological challenges concerning large scale production (Martìn *et al*., 2019; O'Toole *et al*., 2017). Optimization of industrial media for NGPs biomass production is the focus of our project. This aim will be achieved by the valorization and re-use of exhausted fermentation broths derived from traditional probiotics biomass production.

2. PhD Thesis Objectives and Milestones

The project is related to a collaboration within the University of Milan and Lesaffre, one of the largest suppliers of high-performance nutrients designed to address the diverse needs of dairy starter (e.g., lactic acid bacteria), fastidious probiotics, aerobic, and anaerobic strains for fermented food or health applications.
NO-WASTE is aimed at i) contributing to the reduction of waste and environmental impact derived from the microbial biomasses production and ii) recycling of fractions of the spent medium used for biomass production of lactic acid bacteria, considered as "producers", to improve the growth of NGPs, considered as "recipients".
Therefore, the main activities of the project are the following:
A1) Deciphering the primary metabolism of producers and nutritional requirements of recipients
Metabolic prediction-reconstruction of the primary metabolism of 7 species of lactic acid bacteria (*Streptococcus thermophilus, Lactobacillus acidophilus, Lacticaseibacillus paracasei, Lacticaseibacillus rhamnosus*,

Bifidobacterium animalis subsp. *lactis, Lactobacillus brevis* and *Lactobacillus plantarum)*; metabolic prediction-reconstruction of nutrient requirements of 4 NGPs species (*Akkermansia muciniphila, Faecalibacterium prausnitzii, Clostridium tyrobutyricum,* and *Eubacterium hallii*).

A2) Biomass production of different species of traditional probiotics, for exhausted media recovery

Biomasses of *S. thermophilus, L. acidophilus, L. paracasei, Bifidobacterium animalis* subsp. *lactis*, *L. rhamnosus, L. brevis* and *L. plantarum* will be produced at the University of Milan and Lesaffre laboratories using 1L-bioreactors.

A3) Chemical analyses and composition of producer's spent growth media

Task 1. Spent media of producers will be analyzed to determine their composition. We will focus our attention on the quantification of specific metabolites as residual sugar, organic acids, free amino acids (or peptides, proteins, and other nitrogen sources), vitamins, and nucleotides, potential ingredients of an industrial medium for NGPs biomass production. Methods that will be used for metabolomic studies are HPLC, mass spectrometry, MALDI-TOF, NMR.

Task 2. Identification and purification of fractions to be used as additive for the formulation and optimization of new industrial medium for the recipient's biomass production. Based on the composition of the spent medium, molecules will be separated through different centricon centrifugal filter devices at cut-off established based on the molecules of interest to be purified (3-100 kDa). This system will be applied for small volume of medium (1-100 ml). For a scale-up at laboratory level (up to 1 liter), macro- and micro-solutes will be separated by ultrafiltration and by Tangential Flow Filtration Membrane (TFF) (for scale-up towards higher volumes of spent media): permeate and retentate will be recovered after TFF and used as ingredient or additive for NGPs biomass production.

A4) Enrichment-formulation of the recipient's growth media for biomass production

Evaluation of media enrichment on recipient's biomass yield: specific fraction of the producer's spent media will be added to the recipient's growth medium at different amount. Then, different parameters will be evaluated to assess the effect of the new ingredient on the NGPs biomass yield in comparison with the in-use industrial medium. The parameters i) final cell density and ii) viability will be evaluated by flow cytometry and by standard plate counting. Also, iii) morphology will evaluated by flow cytometry and by microscope analysis.

A5) Process scale-up process

After media optimization at lab scale (up to 1L-bioreactor), the scale up at industrial level will be carried out in collaboration with Lesaffre.

A6) Data analysis, manuscripts and PhD thesis preparation

Table 1 *Gantt diagram for PhD thesis project.*

Activities \ Months	3	6	9	12	15	18	21	24	27	30	33	36
A1) Deciphering the primary metabolism of producers and nutritional requirements of recipients	■	■	■	■	■	■						
A2) Biomass production of different species of traditional probiotics, for exhausted media recovery				■	■	■	■					
A3) Chemical analyses and composition of producer's spent growth media					■	■	■	■				
A4) Enrichment-formulation of the recipient's growth media for biomass production							■	■	■	■	■	
A5) Process scale-up									■	■		
A6) Data analysis, manuscripts and PhD thesis preparation	■	■	■	■	■	■	■	■	■	■	■	■

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Innovative bioprocesses and extraction techniques of high value molecules from agricultural biomasses

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The aim of this PhD project is the recovery of value-added compounds from agricultural by-products by applying an integrated approach between fermentation and aqueous mild extraction processes. To reach this goal, after a preliminary proximate composition analysis of by-products, lactic acid fermentation will be applied to produce antimicrobials, aroma compounds and biofertilizers and mild extraction techniques will be used to recover proteins, fats and dietary fibres.

Sviluppo di bioprocessi e tecniche di estrazione di molecole ad alto valore da biomasse agricole residuali

Questo progetto di tesi di dottorato mira al recupero e alla valorizzazione di composti con un valore aggiunto da sottoprodotti della produzione agricola mediante l'applicazione di un approccio integrato che prevede l'uso della fermentazione e di processi di estrazione in fase acquosa o con l'ausilio di enzimi. A questo fine, la fermentazione sarà applicata per produrre composti con attività antimicrobica, aromi e biofertilizzanti, mentre processi di estrazione acquosa verranno usati per recuperare proteine, grassi e fibre.

1. State-of-the-Art

Agricultural wastes and by-products are a well-recognized worldwide problem, affecting the economy, society and the environment. According to FAO (Food and Agriculture Organization of the United Nations) about 1.3 billion tons of food is lost or wasted per year, and fruit and vegetables are the largest food loss contributor. Their actual management strategies are not considered sustainable and have environmental implications. In addition, agrifood waste/byproducts biomasses (e.g.: husk, seeds, stems, roots, pulp, bagasse, and peels) are still rich in value-added molecules, which can be recovered and valorized using bioprocesses and green extraction techniques (Gómez-García et al., 2021).
Solid State Fermentation (SSF) is an ecologic process used for synthesizing value-added compounds using low energy and water while generating minimal waste. The use of fermentation to upcycle by-products is based on the different abilities of microorganisms to convert the constitutive organic compounds into primary and secondary metabolites that can enrich the bioactive content of the biomass or can be extracted from it.
Among these compounds, the present work will be focused on the obtainment of antimicrobials, aroma compounds and biofertilizers from the fermentation of agrifood by-products with lactic acid bacteria (LAB). LAB are generally recognized as safe (GRAS) microorganisms characterized by robustness against several stresses, such as low pH or osmotic stress, and high nutrient uptake and simple metabolism. For this reason, they are considered attractive cell factories for industry (Sauer et al., 2017).
The production of valuable compounds is related to strains and species employed in the fermentation and to the characteristics of the fermented substrates. Many LAB can produce metabolites with antimicrobial activity against some of the most common foodborne pathogens during the fermentation of fruit and vegetable by-products (Ricci et al., 2021). Moreover, LAB are capable of metabolic pathways that lead to the formation of certain aromatic compounds and aroma precursors like aldehydes, esters, and metabolites derived from phenolic acids, lipids, peptides and proteins (Hadj Saadoun et al., 2021). The employment of lactic acid fermentation to produce antimicrobials and aroma compounds can open innovative ways of valorization of agricultural biomasses leading to final products which can be targeted at different fields (food, feed, cosmetic). An additional way to recover these by-products can be the conversion to value-added biomasses to be used as biofertilizers. Bacterial species belonging to the genera of *Pseudomonas, Bacillus, Bacteriodes* have been used in the production of biofertilizers from food by-products (Areeshi, 2022) but the development of steered lactic acid fermentation processes to convert agrifood by-products into biofertilizers is barely explored.
In addition, the recovery of value-added molecules can be also approached by using mild extraction techniques. Enzyme-assisted extraction (EAE) is a specific and efficient method, that works under mild conditions and has a low impact on the environment. This extraction technique takes advantage of the ability of enzymes to hydrolyze cell wall components that allows to reduce the requirement of chemical solvents and heat, promotes the extraction of the desired biomolecules and preserves their structure and functions. However, by using proteases, the protein

fraction can be isolated as a mixture of peptides and free amino acids. EAE has been applied on different vegetal matrices, such as fruits, legumes (Fuso et al., 2022, Prandi et al. 2021). The protein fraction from agricultural by-products can be also isolated by applying direct aqueous extraction (DAE) technique, which consists of extraction with buffer, followed by a separation step. The main advantage is that direct aqueous extraction doesn't need organic solvents, high temperatures, or extreme pH and can be used for the extraction of proteins in different matrices (Prandi et al., 2021). The products obtained with these techniques are strictly related to the proximate composition and the characteristics of the employed by-products. These value-added biomolecules can be applied in food and non-food sectors, generating value from discarded by-products. In addition, following the principles of the circular economy, the work of the PhD project will also focus on the possible implementation of sequential processes to create a performant "cascade biorefinery approach" for the valorisation of agricultural biomasses.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Pre-treatment of by-products:** different pre-treatments (milling, homogenization, etc.) will be applied on a collection of agricultural sidestreams that will be set up during this project, accordingly to seasonality and market availability (e.g., kiwi, Brassicaceae and Brassicaceae leaves, tomato, hop, and many more). These techniques will prepare the matrices for the next steps.

A2) **Chemical composition analysis:** a chemical composition analysis will be done on the different agricultural sidestreams. This step is mandatory for the next ones.

A3) **Design of fermentation processes:** lactic acid fermentation will be applied to recover antimicrobials, aroma compounds and biofertilizers from agrifood by-products. To better understand the effect of the fermentation process it will be studied with the help of experimental designs (DoE) from MODDE® Pro v. 13.0.0. Software (MKS Umetrics, Umeå, Sweden).

A4) **Mild extraction:** the use of different enzymes and buffer-aqueous solutions will be tested in order to promote the extraction of valuable compounds from the residues selected in A1. Reaction will be first optimized at a laboratory scale and then the most promising in term of yield will be applying for a scaling up.

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1. *Gantt diagram for this PhD thesis project*.

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Pre-treatment***																								
A2)	***Proximate composition analysis***																								
A3)	***Design of fermentation processes***																								
	1) Aroma compounds																								
	2) Biofertilizers																								
	3) Antimicrobials																								
A4)	***Mild extraction***																								
	1) EAE / DAE																								
	2) Scale-up evaluation																								
A5)	***Thesis and Paper Preparation***																								

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Fuso A, Viscusi P, Larocca S, Sangari FS, Lolli V, Caligiani A (2022) *Protease-Assisted Mild Extraction of Soluble Fibre and Protein from Fruit By-Products: A Biorefinery Perspective,* Foods 12, 148. https://doi.org/10.3390/foods12010148

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Pigmented wheat as a valuable raw material to produce cereal-based foods with high nutritional value and rich in bioactive compounds

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This PhD thesis project aims to enhance the bioactive potential of pigmented durum and soft wheat varieties through anthocyanin-rich flours production. Appropriate recombination of pigmented wheat milling fractions will allow the production of flours enriched in bioactive compounds useful to obtain innovative functional foods, such as fresh or dry pasta and bakery products that meet the nutritional, sensory, and health needs of consumers.

Il frumento pigmentato quale preziosa materia prima per la produzione di alimenti a base di cereali ad alto valore nutrizionale e ricchi di composti bioattivi

Questo progetto di tesi di dottorato mira a valorizzare il potenziale bioattivo di varietà di frumento duro e tenero pigmentato attraverso la produzione di sfarinati ricchi in antociani. Mediante opportune ricombinazioni di frazioni di macinazione si produrranno sfarinati ricchi di composti bioattivi utili all'ottenimento di alimenti funzionali innovativi, come pasta fresca o secca e prodotti da forno, in grado di soddisfare il consumatore sotto il profilo nutrizionale, sensoriale ed eventualmente salutistico.

1. State-of-the-Art

Anthocyanins are a class of polyphenols responsible for the red, purple, orange, and blue colors of many fruits, vegetables, flowers, and other plants. Scientific research is evaluating the use of anthocyanins in producing foods that may positively affect health (Gupta et al., 2021). Anthocyanins are also found in a few pigmented wheat varieties as polyhydroxylated and methoxylated heterosides derived from the flavylium ion or 2-phenilbenzopyrilium (Figure 1).

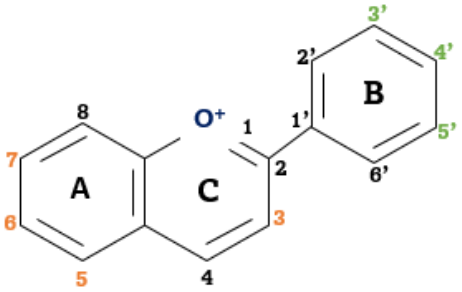

Figure 1 *Flavylium cation with two aromatic rings (A and B) and an oxygenated heterocycle (**C**)*

In pigmented wheat, anthocyanins are localized in the outer layer of the caryopsis, where they preserve the integrity of the plant cells. Scientific literature reports that this class of compounds is not only responsible for the pigmentation of plants but can also exert various protective effects. These properties include antioxidant and anti-inflammatory activities, prevention of heart disease, anti-ageing effects, and improving gut health (Zhu, 2018). Based on the notion that cereal-based products can be suitable systems for delivering bioactive compounds (Ficco et al., 2014), the combination of bioactive molecules, such as anthocyanins, with one of the world's most consumed cereals, such as wheat, makes the latter an ideal raw material from which to derive various functional foods. To date, there is limited evidence on the formulation of anthocyanin-enriched products from pigmented wheat, such as cookies (Pasqualone et al., 2015), bread (Bartl et al., 2015), and dry or fresh pasta (Ficco et al., 2016), and furthermore, these studies still reveal some limitations and highlight the sensitivity of anthocyanins to different process parameters like temperature, light, humidity, and pH. Starting from these issues, this project aims to overcome the limitations by characterizing all fractions obtained by milling pigmented wheat varieties through their chemical and technological properties and anthocyanins content. In this way, it will be possible to identify the most suitable recombination to obtain an enriched flour useful to produce innovative grain-based functional foods with significant nutritional, technological, and health potential through bioactive compounds.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities, according to the Gantt diagram given in Table 1:

A1) **Bibliographic research:** analysis of scientific literature and publications (1-36 months).

A2) **Screening and characterization of pigmented grains:** Screening of durum and soft wheat varieties with high anthocyanin content (A2.1) (4-8 months) on which analysis to determine the proximate composition, anthocyanin content, and other parameters used for their exhaustive characterization will be conducted (A2.2) (6-12 months).

A3) **Grain milling and chemical characterization of milling fractions:** pigmented wheat fractions will be produced using pilot milling plants (A3.1) (12-16 months), and will be evaluated for nutritional composition, along with anthocyanin content (A3.2), to obtain the necessary information for recombination (12-16 months).

A4) **Anthocyanin profile evaluation of milling fractions by chromatographic and spectrophotometric methods:** the individual fractions anthocyanin profile will be evaluated by means of different analytical procedures (A4.1) (12-18 months).

A5) **Milling fractions recombination and flours rheological assessment:** proper recombination of milling fractions to obtain anthocyanin-enriched flours on which the main compositional and rheological parameters (Chopin's alveograph, Brabender's farinograph and micro-viscoamilograph, and Falling number) (A5.1) will be evaluated to identify their potential technological implications (18-20 months).

A6) C**ereal-based anthocyanin-rich foods production**: use of enriched flour to produce innovative functional foods with balanced rheological and health outcomes related to anthocyanins. The innovative products will be characterized by considering nutritional, sensory, and health features (22-36 months).

A7) **Writing and Editing** of the PhD thesis, scientific papers, and oral and/or poster communications (1-36 months).

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		12	14	16	18	20	22	24	26	28	30	32	34	36
A1)	***Bibliographic research***													
A2)	***Screening and characterization of pigmented grains***													
	1) Screening and sampling													
	2) Chemical and technological evaluation													
A3)	***Grain milling and chemical characterization of milling fractions***													
	1) Milling with plant pilot													
	2) Fraction's chemical analysis													
A4)	***Anthocyanin profile evaluation of milling fractions***													
	1) Chromatographic and spectrophotometric method identification													
A5)	***Milling fractions recombination and flours rheological assessment***													
	1) Rheological assessment of flour													
A6)	***Cereal-based anthocyanin-rich foods production***													
A7)	***Thesis and Paper Preparation***													

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Rapid analytical assessment of aroma and visual quality on food products of animal origin

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This PhD research project aims to characterize food products of animal origin (namely poultry and beef meat, dairy products and honey) and assess their quality by sensory and instrumental analytical approaches. More specifically, the main activities will be focused on the evaluation of the aromatic profile and visual aspects by developing and applying rapid, non-destructive and user-friendly analytical techniques, with the final goal to investigate the effect of the farming system, as well as the contribution of the origin, on the quality traits of food products of animal origin.

Valutazione rapida analitica dell'aroma e della qualità visiva dei prodotti alimentari di origine animale

Questo progetto di ricerca di dottorato si propone di caratterizzare alimenti di origine animale (carni avicole e bovine, prodotti lattiero-caseari e miele) mediante analisi di tipo sensoriale e strumentale. In particolare, la valutazione del profilo aromatico e della qualità visiva, mediante la messa a punto ed applicazione di determinazioni analitiche rapide, non distruttive e di facile impiego, rappresenteranno il focus principale del progetto di dottorato, con l'obiettivo finale di indagare l'effetto del sistema di allevamento, nonché l'origine, sui tratti qualitativi e microbiologici dei prodotti alimentari di origine animale.

1. State-of-the-Art

Generally, descriptive sensory tests for food quality evaluation are conducted by trained panels with expert assessors. Although this approach is among the most commonly used it shows various disadvantages, such as being time-consuming and expensive (Chiofalo *et al*., 2017). In this regard, in recent years, human sense perception has been combined with "artificial senses"-based instruments, which have been applied in the food industry for e.g., quality control, freshness and maturity monitoring, shelf-life study and authenticity evaluations. Such equipment may show many advantages, such as being rapid, efficient, low cost and non-destructive, as well as more and more environmentally sustainable (Ali *et al*., 2020). Novel artificial sensing devices, such as e-noses and e-tongues based on hybrid or electronic sensors, are being investigated. The electronic nose usually comprises nonselective sensors that interact with volatile molecules; upon interaction, a signal, constituting a sort of fingerprint of the smells, is produced and used to identify the odour through comparison with a reference library of previously obtained measurements of known samples (Calvini *et al*., 2022). The electronic nose has been used to assess the quality of meat products (Munekata *et al*., 2023), verify the authenticity of Parmigiano-Reggiano (Chiofalo *et al*., 2017), and identify the botanical origin and evaluate the quality of honey (Huang *et al*., 2015). In addition to sensors-based e-nose, other analytical techniques are used for the determination of volatile organic compounds (VOCs), such as solid-phase microextraction with gas chromatography coupled with mass spectrometry (SPME-GC-MS), also associated with multivariate statistical analysis (Calvini *et al*., 2022) or flash gas chromatography (Wang *et al.,* 2022). Another rapid non-destructive instrument is the computer vision system (CVS) (also called as "electronic eye"), which consists of an illumination device, a camera, and a computer with a high-resolution monitor. CVS applications are mainly used for those food products for which appearance is among the main key quality attributes evaluated by consumers (Chiofalo *et al*., 2017). In fact, CVSs can be effectively used for classifying food products into specific grades, detecting visual defects and estimating properties such as colour, shape, size, surface defects and contamination; examples on food products of animal origin are the estimation of fat content in poultry products (Chmiel *et al*., 2011), the prediction of colour grade in beef meat (Sun *et al.*, 2011) and the characterization of several types of honey with different botanical origin (Shafiee *et al*., 2014). Especially, it is relevant to ascertain the eventual effects of farming systems and origin on the main quality traits and composition of meat and dairy products (El-Deek *et al*., 2016). Given the above, the application of instrumental analysis also to corroborate the results obtained by sensory analysis may be of particular interest. In this framework, the research activities of this PhD project will be focused on investigating the effect of the farming system, as well as the contribution of the origin, on the quality traits of different food products of animal origin. To achieve this goal, rapid analytical techniques to evaluate the aroma and the visual quality of such food products will be developed and implemented. In addition, the findings obtained by these methods will be combined with those obtained by conventional instrumental approaches, as well as both descriptive and affective sensory tests, and microbiological analyses. This PhD thesis is part of Alma Idea 2022 project INARIM and European project H2020 INTAQT (INnovative Tools for Assessment and Authentication of chicken meat, beef and dairy products' QualiTies).

2. PhD Thesis Objectives and Milestones

The aim of this PhD project is to assess the quality of different food products of animal origin (namely poultry and beef meat, dairy products, and honey), with a focus on the evaluation of the aromatic profile and visual aspects, through the development and subsequent application of rapid, non-destructive and easy-to-use analytical approaches to investigate the effect of the farming system, as well as the contribution of the botanical origin. Also, some microbiological aspects will be considered. In addition, since these do not require the use of reagents or solvents, they can be considered environmentally and operator health friendly, thus representing a sustainability advantage over other conventional analytical approaches. The project implementation will require the future following activities reported in the Gantt diagram below (Table 1).

A1) Bibliographic research: research in the literature on the image analysis, the assessment of aromatic profile of food products of animal origin, as well as sensory analysis.

A2) Aroma and visual analysis of food products of animal origin: HS-Flash GC, SPME-GC-MS and electronic eye to characterize food products (dairy and meat products, and honey).

A3) Sensory evaluation of food products of animal origin: descriptive analysis (e.g., QDA®, Flash Profile, etc.) of cheese, milk and meat, as well as consumer tests on cheese and meat.

A4) Microbiological analysis of food products of animal origin: microbiological analysis on food products of animal origin, including cheese.

A5) Statistical analysis: univariate and multivariate analysis. Joint statistical analyses of the results obtained from sensory, microbiological tests and instrumental analysis.

A6) Writing of the oral and/or poster communications, scientific papers and PhD final thesis.

Table 1 Gantt diagram for the future activities of this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Bibliographic research***																								
A2)	***Aroma and visual analysis***																								
	1) Set up of the analytical methods																								
	2) Methods application on food products																								
A3)	***Sensory evaluation on food products***																								
	1) Descriptive analysis																								
	2) Consumer test																								
A4)	***Microbiological analysis***																								
A5)	***Statistical analysis***																								
	1) Data elaboration																								
	2) Univariate and multivariate approaches																								
A6)	***Thesis and Paper Preparation***																								

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Experimental strategy for the improvement of the resistance to Common Bacterial Blight (CBB) in common bean (*Phaseolus vulgaris*)

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This PhD research project is aimed at better characterising the resistance to CBB in common bean, in order to facilitate the breeding process. The experimental approach will combine the development of new molecular markers associated with the resistance and a reverse genetic approach that aims at identifying genes involved in the resistance.

Strategia sperimentale per la progettazione ottimale di unità di ultrafiltrazione per il recupero di biopolimeri di interesse alimentare

Questo progetto di tesi di dottorato mira ad aumentare le conoscenze in merito alla resistenza alla malattia CBB in fagiolo con l'obiettivo di facilitare il miglioramento genetico della resistenza. L'approccio sperimentale scelto consisterà sia nello sviluppo di nuovi marcatori molecolari associati alla resistenza che nello studio a livello molecolare della resistenza, tramite un approccio di genetica inversa.

1. State-of-the-Art

Common bean (*Phaseolus vulgaris L.*) is one of the most important grain legume crops worldwide, with over 33 Mha cultivated in 2019 and a production of about 29 million tons (FAOSTAT, 2019). Global common bean production is affected by major biotic stresses: common bacterial blight is one of the most serious diseases of beans, is endemic to most regions where common bean is cultivated and can cause severe yield reduction, even higher than 40% (Singh and Miklas 2015). The bacterial disease is seed-born and caused by *Xanthomonas phaseoli* pv. *phaseoli* and *Xanthomonas citri* pv. *fuscans* (Tugume et al., 2019). Different strategies can be used to manage the disease, these go from cultural practices, such as crop rotation and use of pathogen-free seeds to chemical control, however the preferable method is the use of resistant or tolerant genotypes.
In common bean, the study of CBB resistance has been ongoing for several years and allowed the identification of at least 27 quantitative trait loci (QTLs) for CBB resistance (Chen et al., 2021). At the same time, breeding of common bean for resistance to CBB has been continuously performed over the last 50 years, and cultivars/breeding lines were obtained either from traditional method, using pathogen inoculation and disease screening or by marker-assisted selection (MAS), using three major QTLs linked with the SCAR markers SAP6 on Chr 10, SU91 on Chr 08, and BC420 on Chr 06 (Singh and Miklas, 2015). The marker BC420 has to be avoided in many bean market classes because it is linked with the V locus for seed color (Mutlu et al., 2005), which causes darkened hues, streaks, and spots, making the seeds non-commercially viable.
The breeding programs were successful in generating cultivars showing a certain degree of resistance to CBB, however they lack a complete resistance to the disease (Viteri and Singh, 2014). Furthermore, cultivars with high levels of combined resistance to both less and highly aggressive bacterial strains are lacking as well as cultivars resistant at all plant aerial parts (leaves, flowers and pods) (Singh and Miklas, 2015). Despite the high numbers of QTLs identified, it emerges the necessity to develop new markers to be used for MAS, this is especially true given the variation of resistance observed in cultivars sharing the same markers associated with the resistance (Viteri and Singh 2014). Despite the several years of work, no major resistance genes were molecularly characterised in common beans (Chen et al., 2021). This lack of information is likely due to the difficulties encountered in common bean transformations, which make the functional validation of genes challenging (Hnatuszko-Konka et al., 2014). A possibility in this regard will be the use of a mutagenized population for targeted induced local lesions in genomes (TILLING) for identifying mutants with improved resistance (Fanelli et al., 2021).

2. PhD Thesis Objectives and Milestones

In light of the present state of the art, this PhD project have the following general objectives:
- the selection of the best inoculation method to distinguish between different levels of resistance to CBB;
- the obtainment of new molecular markers to be used for breeding applications;
- the obtainment of mutants of candidate genes in a mutagenized population.

This PhD project will benefit both the private sector, by providing new information for breeders but also the

scientific community by advancing the knowledge of the molecular mechanisms involved in the resistance.

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Test of inoculation methods and generation of segregating population.**
Different inoculation methods will be tested and the most effective in differentiating the susceptibility of common bean germplasm will be selected (A1.1). The segregating populations for the mapping will be obtained by crossing resistant and susceptible genotypes, this activity has been initiated during the first year of PhD (A1.2).

A2) **Identification of new molecular markers associated with the resistance in mapping populations.**
This activity will consist in the generation of a consensus linkage map to identify molecular markers potentially linked to the resistance (A2.1), the segregating populations (F2) will be evaluated for the resistance and analysed for the recombination of the molecular markers (A2.2). The association mapping will produce new markers associated with QTLs (A2.3).

A3) **Reverse genetic approach for identifying the genes responsible for the resistance.**
A set of candidate genes will be identified either by looking at RNA sequencing experiments in genotypes with different susceptibility or by identifying orthologues of S genes in other species (A3.1). A sequencing approach will be used for identifying mutants of the selected genes in a (M2) chemically mutagenized population (A3.2). The mutants will be tested for the resistance to evaluate the involvement of the selected genes in the process (A3.3).

A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Set-up of Population and Inoculation***																								
	1) Inoculation methods																								
	2) Segregating population																								
A2)	***Molecular markers resistance***																								
	1) Consensus map																								
	2) Recombination and resistance																								
	3) Association mapping																								
A3)	***Reverse genetic approach***																								
	1) Candidate genes identification																								
	2) Sequencing of population																								
	3) Resistance analysis in mutants																								
A4)	***Thesis and Paper Preparation***																								

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Digital solutions for on-farm crop quality assessment

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This PhD thesis project is aimed at the use of innovative and digital technologies used in Agriculture 4.0, such as sensors and data analysis tools, to assess and enhance crop yield and crop quality on open fields.

Soluzioni digitali per la misura della qualità delle produzioni agrarie in pieno campo

Questo progetto di tesi di dottorato è volto all'utilizzo di tecnologie innovative e digitali utilizzate nell'Agricoltura 4.0, quali sensori e strumenti di analisi dati, per il monitoraggio e la valutazione della qualità delle produzioni agrarie in campo.

1. State-of-the-Art

Recently, the increasingly extreme weather events due to climate change, such as drought and heavy precipitation and hot and cold waves, as well as environmental pollutions, soil degradation and scarcity of natural resources have negatively affected crop productivity (Araújo *et al.*, 2021). If we add to this that the world population is predicted to rise 31% by 2050, but arable land will be declined by approximately 50 million hectares (Abbasi *et al.*, 2022), strong technological innovation in agriculture becomes important in order to ensure food security. Agriculture 4.0 (or rather 5.0), which aims to sustain the raising food demand in a sustainable way, is the latest evolution of Precision Agriculture (or rather, precision predictions) with the technologies of Industry 4.0. Modern technologies, such as sensors, robotics, Internet of Things (IoT), cloud computing and data analytics are the agriculture 4.0 core technologies and are expected to enhance crop production by collecting, processing, managing, and sharing data (Araújo *et al.*, 2021). Thanks to the use of IoT devices, improvements at every stage of primary production are achieved, starting from the real-time, automatic monitoring of desired parameters in the field by different type of sensors and their further storage and processing. Next, based on the results of data analysis a control system is usually activated to modify the farming practices. Furthermore, by monitoring and storing environmental and crop conditions over time, data analysis tools, such as Artificial Intelligence (AI), Machine Learning (ML) and platforms like decision support system (DSS) can help the end-users in planning farm practises and type and number of farm inputs effectively (Abbasi *et al*., 2022; Araújo *et al.*, 2021; Raj *et al.*, 2021).
One of the main advantages of Agriculture 4.0 is, therefore, the reduction of the waste and water, as well as an efficient use of farm inputs, such as soil nutrient or pesticides. Nevertheless, by predicting production and its quality and by managing agricultural practices with AI, better and higher production should be ensured (Silveira *et al.*, 2021).
Based on the foregoing, irrigation is clearly a key agricultural practise in which IoT-devices are employed to efficiently manage scarce water resources and to achieve high quality production. New smart irrigation systems, by measuring specific parameters through sensors (weather and soil) and analysing the data collected, can automatically control actuators as required (Hamami and Nassereddine, 2020).
Fertilization is another agricultural practise that benefits from the application of IoT technologies since the exact amount and the type of minerals that are necessary in a specific location can be calculated and spread for an optimal plant development and an enhanced quality of its production. However, several other agricultural issues can be performed with IoT-devices, among which crop pest and disease control and phenotyping (Abbasi *et al.*, 2022; Araújo *et al.*, 2021).
Therefore, this PhD thesis will be focused on the application of IoT-devices (*e.g.*, wireless sensor networks) on field for the crop quality assessment. Different type of sensors for non-destructive analyses on crops and their production will be applied to assess their quality in response to environmental changes and different agricultural practises during the growing season. Furthermore, by monitoring and controlling the field conditions crop quality and yield will be enhanced in a sustainable way.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Experimental trial** on open field with real time monitoring of plant parameters of interest and control of agronomic practices with IoT-devices, crop quality assessment during the ripening stages and harvesting of crop production for its further analytical characterization.

A2) **Characterization of crop production** with analytical methods to verify parameters measured with different type of sensors.
A3) **Data processing** to identify changes in yield and quality of production in response to agronomic management and environmental conditions and, to establish correlation between field and laboratory measured values.
A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) ***On-farm experimental trial***																								
A2) ***Characterization of crop production***																								
A3) ***Data processing***																								
A4) ***Thesis and Paper Preparation***																								

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Antifungal activity of bioactive molecules isolated from agricultural waste against rice blast fungus

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This Ph.D. project aims to search for antifungal molecules, which will be extracted from agricultural waste to control rice blast pathogen, *Pyricularia oryzae* Cavara. As strobilurin-fungicide resistance is becoming an urgent problem in rice blast management, both the strobilurin-resistant as well as sensitive strains will be evaluated. Moreover, the project aims to get an insight into the molecules' mode of action, to identify new potential molecular targets for the development of innovative and environment-friendly fungicides, which could control also the strains that developed resistance to currently used synthetic fungicides.

Attività antifungina di molecole bioattive isolate da scarti della filiera agricola, nei confronti del brusone del riso

Questo progetto di dottorato mira ad identificare molecole con attività antifungina nei confronti delbrusone delriso, *Pyricularia oryzae* Cavara, estratte da scarti della filiera agricola. Poiché resistenza alle strobilurine sta diventando un problema urgente nella gestione del brusone del riso, sarà valutata l' attività delle molecole selezionate sia su ceppi sensibili che su ceppi resistenti alle strobilurine. Inoltre, nell'ambito del progetto verrà studiato il meccanismo d'azione delle molecole ottenute, per identificare nuovi potenziali target molecolari per lo sviluppo di fungicidi innovativi e eco-compatibili, che potrebbero essere attivi anche nei confronti dei ceppi che hanno sviluppato resistenza ai fungicidi sintetici attualmente utilizzati.

1. State-of-the-Art:

Fungicide resistance in plant pathogens is spreading mainly because of monoculture cropping and extensive use of synthetic fungicides. It is a global threat to crop security, urgently requiring research focusing on identification and development of environmentally friendly compounds with novel modes of action (Steinberg *et al*., 2020; Piotrowski *et al*., 2015; Pinna *et al*., 2023). One of the most destructive fungal pathogen is the *Pyricularia oryzae* Cavara, a complex pathogen having different host-specialized pathotypes, which infect different important crops (Gladieux *et al.,* 2018). The most common is the rice-pathotype, causing severe blast disease in rice. Other crops, such as wheat, are also affected by its host-specialized pathotypes. Wheat blast has been reported in South America and South Asia. *P. oryzae* is also responsible for the grey leaf spot disease of turf grasses.
In rice, the disease causes annually 10-30% losses (Kunova *et al.,* 2021). The pathogen can infect rice plant at all growth-stages, but the major damage is caused by infection of the last internode (neck blast) and the panicle. Moreover, secondary metabolites such as pyriculols, nectriapyrones, tenuazonic acid (TeA) are produced by *P. oryzae,* of which TeA is the most toxic, showing acute toxicity to mammals and also has inhibitory activity of photosynthesis (Motoyama *et al.*, 2020).
Rice blast management is based on integrated management, but fungicides still represent the most common approach. Strobilurins are well-consolidated fungicides and among the most widely used, especially in the European risiculture. Their almost exclusive use, and a single-site mode of action are important factors for quick development of resistance in pathogen populations (Kunova *et al.,* 2021). The scarcity of alternative fungicides effective against rice blast highly increases the risk of QoI resistance development in Europe, and in particular in Italy. Molecular studies of the cytochrome b (cytb) – the target of QoI fungicides – identified the presence of the G143A mutation responsible for the QoI resistance also in Italian isolates (Tenni *et al*., 2021) accelerating the urgency of the development of novel means for the disease management . Recent research indicates that often the extract obtained from agricultural waste contain a set of bioactive molecules with antifungal activity. Extract from agro-industrial wastes of unripe grapes showed antifungal properties against multiple strains of *Candida* spp. and other dermatophytes (Simonetti *et al*., 2019). Extracts from agricultural matrices such as grapevines were reported to be endowed with bioactive molecules with high antifungal activity, e.g. pterostilbene or trans-resveratrol were shown to be active against *Plasmopora viticola* and *Botrytis cinerea* (Guerrero et al., 2016).

These studies indicate that agricultural wastes are a rich source of bioactive molecules with antifungal activity and with novel mechanisms of action.

2. PhD Thesis Objectives and Milestones:

Objectives in the context of this ongoing PhD thesis can be achieved by certain activities subdivided below through the Gantt diagram. A1, A2, and a part of A3 are not included in the Gantt chart as these have been performed in the 1st year.

A1. Extraction from agricultural waste: Different matrices of agricultural wastes were collected followed by extraction by aqueous and organic solvents (e.g. methanol).
A2. Characterization of the extracted molecules: Molecules present in the extract were characterized by HPLC, Mass-spectrometry, possibly using the University of Milan COSPECT platform.
A3. In vitro evaluation of biological activity of extracted molecules: Mycelium inhibition test and spore germination inhibition test was performed to evaluate the efficacy of the crude extracts and purified molecules. Eventually, molecules with the highest biological activity were subjected to enzymatic assays to measure e.g. the mitochondrial activity.
A4. In vivo evaluation of biological activity of the extracted molecules: The preventive and post-inoculation application of the compounds will be evaluated in leaf-disk assay, in greenhouse, and possibly in a field trial to assess the reduction of disease symptoms.
A5. Investigation of the mode of action of the molecules through novel tools: To get an insight into the mode of action of the extracted molecules, transcriptomic analysis will be done to conclude this study. Pathogen treated or not with the compound will be subjected to RNA extraction and then differential gene expression will be analysed to get the hints about the mode of action of the new molecules.
A6. Thesis and Paper Writing: Paper and thesis writing will take place according to the progress of the work.

Table 1 Gannt chart for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A3)	***Enzymatic assay***	■	■	■	■	■																			
A4)	***In vivo trials***	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■							
	1) leaf-disk assays	■	■	■	■	■																			
	2) greenhouse trials						■	■	■	■	■	■	■												
	3) field trials													■	■	■	■	■							
A5)	***Mode of action studies***																		■	■	■	■	■	■	■
A6)	***Thesis and Paper Preparation***	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

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Shelf-life extension of food by using of innovative biodegradable packaging

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This PhD thesis research project is aimed at evaluating the effects of the use of biodegradable packaging, edible coatings and films, possibly added with natural bioactive compounds, on the shelf life of food. A study of the degradation kinetics will be carried out, based on the monitoring of the main qualitative indices, to find effective and sustainable solutions to be used as an alternative to the use of non-degradable plastic polymers, with the aim of prolonging the shelf life of food maintaining high quality and obtaining benefits for the environment and health.

Estensione della shelf-life degli alimenti tramite l'impiego di packaging innovativo biodegradabile

Questo progetto di tesi di dottorato ha lo scopo di valutare gli effetti dell'uso di imballaggi biodegradabili, rivestimenti e film edibili, eventualmente addizionati con composti bioattivi naturali, sulla shelf life degli alimenti. Verrà effettuato uno studio delle cinetiche di degradazione, basato sul monitoraggio dei principali indici qualitativi, al fine di trovare soluzioni efficaci e sostenibili da utilizzare in alternativa all'utilizzo di polimeri plastici non degradabili, con l'obiettivo di prolungare la durata di conservazione degli alimenti mantenendo un'elevata qualità e ottenendo benefici per l'ambiente e la salute.

1. State-of-the-Art

Packaging is an essential element in the food chain as it plays the fundamental role of protecting and preserving the qualitative characteristics of the food product from production to distribution and consumption, delaying the deterioration of the product, extending the shelf-life, limiting food waste and ensuring food safety. Synthetic packaging materials obtained from limited and non-renewable petroleum-based resources such as polyethylene (PE), polypropylene (PP), polyethylene terephthalate (PET), are among the preferred ones due to their mechanical properties, durability over time, ease of processing and cheapest production costs. The intensive use of these materials constitutes one of the most criticized environmental challenges due to the serious consequences on the environment due to their synthetic nature. In addition to the emission of toxic gases during production and disposal, by accumulating in the environment, they lead to the formation of microplastics and chemical substances that damage human health and threaten natural balances (Nilsen-Nygaard *et al*., 2021; Kumar *et al*., 2022).
Nowadays, the consumer is increasingly attentive to health, food safety, sustainability, and environmental concerns. The increased awareness and the change in lifestyles have led to the research and development of sustainable food packaging systems capable to less impact on the environment. Replacing plastic packaging with bio-based and biodegradable materials could help reduce problems such as depletion of natural resources, waste management and disposal, global warming, etc. To meet these expectations, research is focused on the use of biopolymers combination for the formation of biodegradable packaging materials and edible coatings as substitutes for synthetic polymers. Moreover, the possibility to add natural and bioactive substances on the packaging formulation results as a valid opportunity to extend the food shelf life and reduce the food wastes. This new concept of packaging has aroused considerable interest thanks to the advantages related to biocompatibility, economy, and non-toxicity (Kumar *et al*., 2022).
An edible coating solution can be used for the formation of coatings which are formed directly on the surface of the product by dipping or spraying in the form of a thin film which it covers them or films which are produced starting from the solution with the obtainment of a preformed thin layer in which food can be wrapped (Grzebieniarz *et al*., 2023). The application of edible coatings to foods dates to the 12th and 13th centuries when wax was applied to citrus fruit in China. Later, waxes became commercially available as food coatings (Park, 1999).
Currently, biopolymers used for the formation of biodegradable packaging or edible coatings can be classified according to their origin as synthetic such as polylactic acid (PLA) or polybutylene succinate (PBS), microbial such as polyhydroxyalkanoates (PHA) or natural such as polysaccharides, proteins, and lipids (Nilsen-Nygaard *et al*., 2021). The properties of interest of the packaging materials are the gas and water vapor barrier properties, the mechanical resistance, the improvement of the visual aspect and the release of compounds of interest such as antioxidants, antimicrobials, anti-browning, nutrients from additives used in their formulation (Iñiguez-Moreno *et al*., 2021). However, single biodegradable materials have limitations due to poor barrier, mechanical and processing properties compared to conventional polymers. In the current scientific panorama, there are many examples of application of single biopolymers such as biodegradable packaging material and edible film and coating to extend the shelf life of food, some applications show a positive effect on food products while others highlight the criticalities of the single material (Nilsen-Nygaard *et*

al., 2021; Kumar *et al*., 2022).The formation of composite coatings or multilayer packaging can be an effective solution to combine the characteristics of the single polymers obtaining an improvement of the functional properties of the final film also thanks to the addition of bioactive compounds and additives such as plasticizers and emulsifiers that improve flexibility, extensibility and stability of the coating (Iñiguez-Moreno *et al*., 2021; Grzebieniarz *et al*., 2023). Although several works exist on the application of single and multilayer films (Grzebieniarz et al., 2023), more in-depth research is needed on their larger use and on the monitoring of kinetic processes during their application in food preservation.

2. PhD Thesis Objectives and Milestones

This PhD thesis project will be directed to evaluate the possible application of single and composite films and coatings in food preservation. The study of the kinetic processes of deterioration and modifications of the chemical, physical, microbiological, and sensorial parameters of foods during the storage time will be carried out. Different types of innovative and biodegradable packaging will be tested to identify the optimal conditions that allow to define and extend the shelf life. Within the overall mentioned objectives, the current PhD thesis project was divided into the following activities according to the Gantt diagram given in Table 1:

A1) **Individuation of biopolymers for food packaging application** through the study of biopolymers properties from bibliographic research (A1.1), and the analysis of the current application of biopolymers to food on the market (A1.2) with the aim to verify the application to the identified food for this research.

A2) **Development of the formulations and application of innovative packaging to food systems.** Single and combined packaging systems will be evaluated for their stability, physical and mechanical properties (thickness, colour, moisture content, water solubility, texture) to find solutions with better functional proprieties (A2.1).

A3) **Monitoring of the products shelf life** to evaluate the changes in the quality parameters as physical parameters (weight loss, colour, texture analysis), chemical and nutritional (titratable acidity, total soluble solids, water activity, pH, organic acids content, bioactive compounds, antioxidant activity), microbiological and sensorial (flavour, taste, aroma, visual aspect) during the storage time using the different types of innovative and biodegradable packaging (A3.1)

A4) **Definition of the products shelf life** through the study of the degradation kinetics of quality indexes to identify the most suitable packaging systems which allow to extend the shelf life of food maintaining high quality (A4.1)

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Individuation of biopolymers for food packaging application***																								
	1) Study of biopolymers properties																								
	2) Analysis of biopolymers food applications on market																								
A2)	***Application of innovative packaging to food systems***																								
	1) Formulation and characterization of packaging systems																								
A3)	***Monitoring of the products shelf life***																								
	1) Evaluation of quality parameters change																								
A4)	***Definition of the products shelf life***																								
	1) Study of the most suitable innovative packaging																								
A5)	***Editing of thesis and scientific papers***																								

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Capturing the Dynamics of Sensory and Emotional Experience for Sustainable Product Innovation

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This Ph.D. thesis research project is aimed at the development of new methodologies to evaluate innovative products characterized by higher environmental sustainability (e.g., reduced water and other natural resources consumption, new ingredients reducing environmental impact). The "global" experience of a product is considered by integrating the sensory, emotional, and functional dimensions with the setting up of an experimental procedure to capture the changes in sensory perception, liking, and emotional experience of a product during its use.

Catturare le dinamiche dell'esperienza sensoriale ed emozionale per l'innovazione sostenibile di prodotto

Questo progetto di tesi di dottorato è finalizzato allo sviluppo di nuove metodologie per la valutazione di prodotti innovativi caratterizzati da una maggiore sostenibilità ambientale (ad esempio, un ridotto consumo di acqua e altre risorse naturali, o nuovi ingredienti che riducono l'impatto ambientale). L'esperienza "globale" di un prodotto è considerata integrando le dimensioni sensoriali, emozionali e funzionali con la messa a punto di una procedura sperimentale per cogliere i cambiamenti nella percezione sensoriale, nel gradimento e nell'esperienza emozionale di un prodotto durante il suo utilizzo.

1. State-of-the-Art

Sustainable innovation has become a must for companies in the personal care product category and is motivated by business ethics, regulations, trends, and technological growth. Sustainable innovation aims to gradually reduce the negative environmental impact of products during production, use, and disposal, without compromising their quality, sensory, and functional characteristics. To meet consumer interest, new products must present a combination of familiar (reassuring) and novel (attractive) aspects. Although experienced as a whole, a product is a combination of different characteristics: the physical object characterized by a specific sensory identity, the packaging, the brand, and the information communicated on the label. Each aspect that constitutes a product can create expectations that can be confirmed or disconfirmed, provoking different emotional responses. The study of emotions can provide additional information for understanding product performance and optimization, discriminating even deeper than liking. In addition, it can support the development of effective communication, as emotions can be elicited by the product's sensory properties (without considering the brand or other information). In fact, it is important to observe how emotions change in product evaluations under blind-expected-informed conditions to identify the weight of sensory properties and information (Spinelli & Monteleone, 2018). Table 1 lists the main applications of methods to measure emotional responses to food and non-food products, indicating their sensitivity.

Table 1 Main methods to measure emotional responses to food and non-food products.

Methods	Measure	Sensitivity			
Explicit	Verbal and visual Self-reports	Valence	Arousal	Emotion Specificity	
Implicit	Implicit tasks (IAT, AMP, Priming, etc.,)	Valence	Approach/Avoidance		
Psycho-physiological	Autonomic nervous system (fEMG, GSR, HRV, etc.,)	Valence	Arousal		
Brain activity	EEG, FMRI, etc.		Approach/Avoidance		
Behavioral	Vocal characteristics		Arousal	Emotion Specificity	
	Facial and whole-body behavior	Valence			-

IAT: Implicit Association Test; AMP: Affective Misattribution Paradigm; fEMG: Facial ElectroMyoGraphy; GSR: Galvanic Skin Response; HRV: Heart Rate Variability; EEG: ElectroEncephaloGraphy; FMRI: Functional Magnetic Resonance Imaging.

Moreover, in the case of products that require the use, it has been suggested that emotions may vary at different stages,

so the time dimension should be considered. In this sense, the L.U.D. (Learning-Use-Deprivation (Morizet *et al.*,2021)) methodological approach aims to overcome these limitations by considering repeated exposure to the product to arrive at an estimate of consumer adoption of the innovation. L.U.D. is, in fact, an acronym for Learning-Use-Deprivation, or the three phases of which this home test protocol is composed, which considers a phase in which one learns to use the new product (which may often have innovative aspects that do not make it immediately correct for use), a phase of actual use, and an evaluation after a phase in which the product is withdrawn to measure its recall and "lack thereof" (Morizet *et al.*,2021).
While initially the L.U.D. approach was designed for "disruptive" innovations, in this project it will be adapted to the study and support of sustainable, even incremental, innovations.
In addition, the evidence that sensory perception while testing a product is a temporal process was acknowledged by sensory evaluation approximately 60 years ago with the Time-Intensity (TI) technique. More recently, the Temporal Dominance of Sensations (TDS) technique was proposed to fill the gap between static multidimensional sensory profiling and dynamic unidimensional Time-intensity (TI) by offering a way to assess simultaneously several attributes dynamically over time. Emotions are characterized by rapidity of change and relatively short duration. Jager *et al.,* (2014) consequently proposed the Temporal Dominance of Emotions (TDE) as a straightforward extension of TDS, replacing sensory with emotional attributes (Sclich, 2017). This Ph.D. thesis project will be directed to develop new methodologies or adapt methodologies used in the food domain to personal care products to study the change of elicited emotions over time and their relationship with sensory perception: during a single use (with the TDE technique), during repeated uses (L.U.D. framework) and in the memory after use (with studies on Incidental Learning (Mojet & Koster, 2002)).

2. Ph.D. Thesis Objectives and Milestones

Within the overall objective mentioned above, this Ph.D. thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) **Data collection using a multidisciplinary approach** on a minimum of 100 regular consumers of personal care products for each study. In the first year of the Ph.D., Study 1 aimed to develop and optimize TDS-TDE techniques to measure the change in emotions during single product use. In the second year Study 2 will be focused on the L.U.D. framework (A1.1), to measure the change in emotions during repeated uses (before, during, and after use). In addition, in the second-year data collection with the incidental learning procedure (A1.2) will take place to measure the change of emotions in the memorability of a sustainable personal care product (Study 3). These studies will include questionnaire administration to assess socio-demographic, quality-of-life information, psychological traits, attitudes towards personal care products, emotions, preferences, and habits (A1.3).

A2) **Statistical data analyses** with univariate methods to test the effect of the variables, but also with multivariate methods to explore the relationship between sensory properties and emotions, and to identify groups of consumers differing in their individual differences.

A3) **Periods at the Company/abroad** with the aim of collaborating on developing and optimizing methods for measuring emotional responses specifically tailored to different sustainable personal care product innovations.

A4) **Writing and Editing** of the Ph.D. thesis, scientific papers, and oral and/or poster communications.

Table 2 Gantt diagram for this Ph.D. thesis project (2nd and 3rd year).

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Data Collection (Multidisciplinary approach)***																								
	1) L.U.D framework (Study 2)																								
	2) Incidental learning experiment (Study 3)																								
	3) Administration of questionnaires																								
A2)	***Statistical Data Analyses***																								
A3)	***Periods at the Company/abroad***																								
A4)	***Thesis and Paper Preparation***																								

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Innovative technologies for the formulation of bioactive ingredients from plant-based by-products and development of functional foods

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Lipid oxidation is the major challenge when developing functional foods and supplements containing vegetable oils rich in polyunsaturated fatty acids (PUFAs). Despite the recognized nutritional value of PUFAs, their physical and chemical characteristics still limit their application. Microencapsulation of PUFAs rich oils offers the possibility of obtaining lipophilic ingredient with enhanced stability and bioavailability. Accordingly, this PhD thesis project aims to investigate the encapsulation of polyunsaturated oils into different food-grade wall material, using a novel technology based on supercritical carbon dioxide, named particles from gas saturated solution (PGSS).

Tecnologie innovative per la formulazione di ingredienti bioattivi da sottoprodotti vegetali e sviluppo di alimenti funzionali

L'ossidazione dei lipidi è la sfida principale quando si sviluppano alimenti funzionali e integratori contenenti oli vegetali ricchi in acidi grassi polinsaturi (PUFAs). Nonostante il riconosciuto valore nutrizionale dei PUFAs, le loro caratteristiche fisiche e chimiche ne limitano ancora l'applicazione. La microincapsulazione di oli ricchi in PUFAs offre la possibilità di ottenere ingredienti con migliori proprietà funzionali e una prolungata stabilità all´ossidazione. Questo progetto di tesi di dottorato mira ad incapsulare oli ricchi in acidi grassi polinsaturi applicando una nuova tecnologia basata sull´utilizzo dell´anidride carbonica supercritica, denominata "particelle da una soluzione satura di gas (PGSS)".

1 State-of-the-Art

Consumer´s increasing interest in the health enhancing role of specific foods and physiologically active food components is having strong impact on the food industry. One of the major challenges is developing new ingredients that are healthy, functional, and natural (Bharat *et al.,* 2016). Vegetable oils are the main dietary source of essential fatty acids. They play an important role in the body satisfying nutritional needs. They are vital for the normal functioning of the brain and nerve system. The term essential fatty acid refers to polyunsaturated fatty acids (PUFAs) that cannot be produced by the human body and must be obtained from the diet (Kaur *et al.,* 2014). However, PUFAs have an unstable chemical structure and are susceptible to oxidation, isomerization, polymerization, and volatile component loss when exposed to different environmental stresses such as oxygen, light, moisture, and heat. This can lead to the formation of hydroperoxides and results in unpleasant flavours and odours (Jurić *et al.,* 2022). As a result, the health-benefit properties of PUFAs remain underused in formulated food products. Therefore, it is critical to develop suitable methods to improve the oxidative stability of PUFAs during processing and storage.

Microencapsulation is an established strategy for overcoming these challenges. The technology allows to formulate unstable oily molecules into free-flowing and stable powders, reducing oxygen access and providing good oxidation protection for the oil (Reis *et al.*, 2022). Several technologies such as spray drying, freeze drying, coacervation, and extrusion among others are often used to form solid lipid particles. Nevertheless, particle formation employing supercritical carbon dioxide (SC-CO_2) has received increasing attention, as it operates at relatively low temperature and in an oxygen-free environment (Klettenhammer *et al.,* 2020). The Particles from Gas Saturated Solutions (PGSS) process is the most common example of encapsulation technology based on SC-CO_2. The principle consists of saturating the supercritical fluid with the mixture made of the bioactive compound and the wall material. The saturated solution is then rapidly expanded though a nozzle at atmospheric pressure. The rapid release of CO_2 causes an intensive cooling effect leading to the formation of solid or liquid particles. PGSS is a green technology that offers clear advantages, by using a non-flammable, non-toxic, abundant, cheap and generally recognized as safe solvent (SC-CO_2) (Kravanja *et al.,* 2022). Moreover, it is suitable for lipophilic formulations (due to the apolar nature of CO_2), and ensures gentle treatment for heat-sensitive bioactive compounds (Klettenhammer *et al.,* 2020).

2 PhD Thesis Objectives and Milestones

The research objectives of this PhD project can be achieved through the following activities and working plan as shown in the Gantt diagram given in Table 1:

A1) **Microparticles production using PGSS**
To evaluate the effectiveness of PGSS, for designing encapsulated ingredients (polyunsaturated fatty acids rich oils) using combination of different food-grade wall material, and assessing their loading capacity and oxidative stability.

A2) **Co-encapsulation of edible oils with natural (plant by-product) antioxidants**
Although, the encapsulated oil is protected by the wall material, the addition of antioxidants can enhance its oxidative stability and offer advantages in producing powders with a variety of bioactive functionalities. Consequently, vegetable by-products will be extracted using SC-CO_2, characterized, and added to the formulation.

A3) **Application or incorporation of these microparticles in food products**
To understand if the claimed obtained physiochemical and functional properties are transferrable to food products, the encapsulated particles will be incorporated into food matrices to evaluate the functionality of the formulation.

A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 *Gantt diagram for this PhD thesis project.*

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Microparticles production using PGSS***																								
	1) Process optimization																								
	2) Powder characterization																								
A2)	***Co-encapsulation with natural antioxidants***																								
	1) characterization of extracts																								
	2) Oxidative stability assessment																								
A3)	***Incorporation in food products***																								
	1) Effect of processing on the stability of the formulation																								
A4)	***Thesis and Paper Preparation***																								

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Innovative technological applications of plant-based ingredients

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This PhD research project is aimed at developing protein and starch ingredients derived from different plant-based raw materials, promoting the applications of local resources e.g., legumes and cereals and by-products following the main principles of green and circular economy. The ingredients will be produced through the dry fractionation process. Technological, nutritional and sensory characteristics of the ingredients will be investigated to produce innovative food products and pursuing the 12 Goal established by the 2030 Agenda "Responsible consumption and production".

Applicazioni tecnologiche innovative di ingredienti plant-based

Le attività di ricerca saranno volte a sviluppare ingredienti proteici e amidacei, ottenuti attraverso il processo di dry-fractionation, derivanti da diverse materie prime vegetali locali, ad esempio legumi e cereali, nonché scarti e sottoprodotti dell'industria alimentare seguendo i principi di green economy ed economia circolare. Lo studio delle loro caratteristiche nutrizionali, tecnologiche e sensoriali sarà necessario all'impiego in prodotti alimentari innovativi perseguendo così l'obiettivo 12 dell'Agenda 2030 "Garantire modelli di consumo e produzione sostenibili".

1. State-of-the-Art

Today, consumers are shifting toward a healthy lifestyle, promoting ethical and sustainable behaviors. Plant-based foods earn interest in consumers, because the reduction of animal foods is often associated to well-being (Ma et al., 2021). To support this healthy lifestyle, companies formulate innovative foods with high nutritional value using plant-based protein ingredients marked in the form of flours, concentrates and isolates (Ma et al., 2021). These are usually produced through the wet fractionation technique (Ma et al., 2021), which has the advantage of obtaining ingredients with high protein content (70-90%) through the solubilization of the raw material and the precipitation of proteins using chemicals and a drying step to obtain a powdery ingredient. However, the use of chemicals, energy and water, is in contrast with the aim of promoting a sustainable food system (Schutyser et al., 2011).
A sustainable alternative to produce plant-based protein is the dry-fractionation process, which is based on the solely physical separation of the raw material in two fractions. This technique consists in two steps: milling of the raw materials to obtain a micronized flours and separation with an air-classifier to obtain a starch-rich fraction (coarse fraction) and a protein-rich fraction (fine fraction) (Schutyser et al., 2011; De Angelis et al., 2021). This technique is versatile, and it can be applied to legumes, some cereal crops (e.g., wheat and barley) (Schutyser et al., 2011) and to recover and upcycling different industrial by-products (e.g., bran) (Zhang et al., 2019). Future prospects are linked to the improvement of the application to cereals and pseudocereals in which the separation is difficult due to the inhomogeneous starch particle size distribution (Schutyser et al., 2011). Usually, the protein content is lower compared to the protein isolates (e.g., in legumes 55-60%), but the native structure of the protein is preserved, because no chemicals and high temperature are used. In fact, the native structure leads to a different protein performance from the denatured proteins (e.g., foaming, gelling, solubility) (Schutyser et al., 2011; De Angelis et al., 2021). For example, it has been reported that in denatured state some hydrophobic groups are exposed, compared to the protein native state and may influence some functional performance such as solubility and foaming (Tabtabaei et al., 2019). The starch-rich fraction produced through the dry fractionation process usually is considered a co-product of the protein production and is destinated to feed industry (Ren et al., 2021). In fact, the starch used for food production is obtained via wet extraction which presents the same problematics described above (Ren et al., 2021). The starch in food can be used as gelling, binder, stabilizer, and thickening ingredient. Functional performance and food application of protein and starch concentrates obtained through the dry fractionation process have been investigated only in the last few years. Since Apulian region is considered a main Italian region in the production of cereals and legume (Piergiovanni, 2021), dry fractionation technique may be applied to valorise these typical cultivations. Therefore, the aim of this PhD thesis project is to study the protein and starch ingredients obtained through the dry fractionation process. Legumes, cereals and pseudocereals and industrial by-products will be considered as raw materials, in order to promote a responsible production in accordance with the Goal 12 of the 2030 Agenda. Characterization of the ingredients will be necessary to produce innovative foods (e.g., meat, fish or dairy analogues, and egg replacers), promoting the use of local ingredients. The foods produced will have a balanced nutritional profile and will be clean label.

2. PhD Thesis Objectives and Milestones

The PhD thesis project is divided into activities (A) presented in the Table 1 with the aim of set up the dry fractionation process, characterize the protein ingredients that will be used in food production:

A0) Bibliographic research

A1) Application and set-up of the dry fractionation process to local raw materials: by-products, cereals, and legumes: these activities, carried out in collaboration with Innovaprot srl., will be focused on the set-up of the dry fractionation process applied on different raw materials (e.g., legumes, cereals and by-products) (A1.1) and the subsequent characterization of the obtained fractions (A1.2). Specifically, chemical composition, rheological and functional properties will be determined to better understand their possible application in food products.

A2) Set-up of the extrusion process to improve sensorial, nutritional, and functional properties: the aim will be to study the set-up of the extrusion process on the protein and starch fractions (A2.1). The protein fraction will be used to produce textured vegetable proteins (TVP) which will be include as ingredient in food (e.g., meat or fish analogues). The starch fraction will be used to produce ingredients for ready to eat products (e.g., snack). The structured ingredients will be characterized to understand their potentiality in food application (A2.2). This activity will be carried out in collaboration with the Center of Food and Fermentation Technologies (Tallin, Estonia).

A3) Food application of the ingredients and their characterization: the protein and starch ingredients in the form of flour and/or structured products will be used to produce foods (A3.1, A3.2) with the purpose of fortify or replace animal proteins in foods (e.g., egg replacers, meat and dairy analogues). Pasteurized fresh pasta filled with TVP frozen stored will be also produced to study the effect of the thermal treatments and freezing on the TVP and food produced (A3.3, A3.4). All the innovative foods will be characterized in their nutritional, textural and sensorial features. These activities will be carried out in collaboration with Gastronomia Frost Srl (Castellana Grotte, Italia).

Table 1 Gantt diagram for this PhD thesis project.

ACTIVITY/ MONTH		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A0	**Bibliographic research**																								
A1	**Application and set-up of the dry fractionation process to local raw materials: by-products, cereals, and legumes**																								
A1.1	Set up of the dry-fractionation process																								
A1.2	Characterization of dry-fractionated proteins and starch																								
A2	**Set-up of the extrusion process to improve sensorial, nutritional, and functional properties**																								
A2.1	Set-up of the extrusion process																								
A2.2	Nutritional and functional characterization of the extruded raw material																								
A3	**Food application of the ingredients and their characterization**																								
A3.1	Application of dry-fractionated protein and starch in foods and their characterization																								
A3.2	Application of the extruded products to produce innovative foods and their characterization																								
A3.3	Study of the thermal treatment on the qualitative characteristics of the extruded ingredients and foods																								
A3.4	Study of the frozen storage on the qualitative characteristics of the extruded ingredients and foods																								
A4	**Data analysis, thesis and papers writing**																								

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Production of γ-aminobutyric acid by *Levilactobacillus brevis*: basic aspects and applications in foods

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The PhD thesis project is focused on the exploitation of lactic acid bacteria, as bio-factories for the production of bioactive compounds useful for the development of functional foods. Specifically, the production of γ-aminobutyric acid (GABA) by *Levilactobacillus brevis* will be optimised, and the metabolite will be purified and supplemented in several foods. Moreover, the *in-situ* production of GABA will be also evaluated by using a selected *Lvb. brevis* strain as adjunct culture during food fermentations.

Produzione di acido γ-aminobutirrico da *Levilactobacillus brevis*: aspetti basilari e applicazioni nell'industria alimentare

Il progetto di tesi di Dottorato è focalizzato sullo sfruttamento dei batteri lattici, come bio-fabbriche per la produzione di composti bioattivi utili allo sviluppo di alimenti funzionali. In particolare, la produzione di acido γ-aminobutirrico (GABA) da parte di *Levilactobacillus brevis* sarà ottimizzata e il metabolita sarà purificato e aggiunto in diverse matrici alimentari. Inoltre, la produzione *in-situ* di GABA sarà valutata anche utilizzando, come coltura aggiuntiva durante le fermentazioni, un ceppo di *Lvb. brevis* opportunamente selezionato.

1. State of the Art

Microorganisms are a potential source of bioactive compounds (e.g. organic acids, biopeptides, short chain fatty acids, exopolysaccharides, antioxidants, prebiotics) and may be used as cell factories to produce functional foods through the *in-situ* production of bioactive metabolites (directly in food matrices) or by food supplementation with their postbiotics (Nataraj et al., 2020). Among the beneficial microbial metabolites, γ-aminobutyric acid (GABA) is receiving great attention in food, pharmaceutical and cosmetic industries. GABA is a non-proteinogenic aminoacid that may act several physiological functions (e.g. brain development, regulation of neurological disorders, hypotensive, analgesic, antianxiety, antidiabetic effects) that result in proven benefits to human health (Dhakal et al., 2012; Diana et al., 2014; Xu et al., 2017). GABA may be produced via chemical synthesis or through the decarboxylation of glutamate by pyridoxal 5'-phosphate (PLP)-dependent microbial glutamate decarboxylases (Xu et al., 2017). Microbial bioconversion is preferred to the traditional chemical methods because provides food-grade and eco-friendly product that can be used as supplement in fortified and functional foods, as well as in drugs and cosmetics. The global GABA market is expected to grow in the next years and the production by microbial fermentation is expected to exceed that by chemical synthesis. Several microorganisms have been recognised as potential GABA-producers (Dhakal et al., 2012; Diana et al., 2014); among them, lactic acid bacteria (LAB; especially those belonging to *Levilactobacillus brevis* and *Lentilactobacillus buchneri* species) are considered the most promising GABA-producing group (Li and Cao, 2010; Dhakal et al., 2012). The genetic equipment of LAB strains is crucial for the bioconversion of glutamate to GABA, even if other factors, such as pH values, temperatures, time of fermentation, composition of growth substrate and cell density, may affect the functionality of GABA production system. Often, the downstream processes needed for separation and purification of GABA from culture broth are expensive and may impair the large-scale production and the marketability of GABA. Therefore, the optimisation of fermentative processes to ensure high-yield GABA production and the development of low-cost downstream protocols may be of practical relevance. The natural content of GABA in foods (Diana et al., 2014) is low and strategies to increase its concentration are recently gaining interest. However, the production of GABA-enhanced foods is still at experimental levels, and different approaches to incorporate the GABA-producing strains or the purified biocompound in food matrices are critical to ensure the appropriate content and functionality of GABA in supplemented foods. Several authors investigated the use of resting cells (non-growing but metabolically active) for bioconversion processes as they have several advantages compared to growing cells (costs of fermentation process) or purified enzymes (limitations of separation techniques). On the other hand, other studies, have addressed on the use of strategies for the protection of microbial cells and GABA (e.g. immobilization, microencapsulation) to improve their stability in food matrices (Thangrongthong et al., 2020; Ozer et al., 2022). However, these data, although promising, are still preliminary and further studies are needed for the development of GABA-functionalized foods, able to meet the needs of the market and consumers. Based on the above considerations, the aim of PhD project will be the optimisation of GABA production (by testing and selecting appropriate conditions in bioreactor cultivation), as well as of extraction and purification methods. GABA-producing strains or the purified biocompound will be also use to develop potential functional foods and/or

beverages, with enhanced GABA content.

2. PhD Thesis Objectives and Milestones

The PhD project will be divided in several activities, as described below and in the Gantt diagram (Table 1):

A1) **Optimization of biomass and GABA production**: GABA production by *Lvb. brevis* strains will be optimised in controlled conditions using cultivations in bioreactor (A1.1). Biomass will be used for GABA production in different buffer systems and glutamate/GABA content will be measured in both low-cost media and buffers. Chromatographic techniques will be used for detecting glutamate consumption and GABA production (A1.2). Gene expression (RT-qPCR) will be carried-out to elucidate the bioconversion mechanisms and regulation pathways.

A2) **Separation and purification of GABA from synthetic media and buffer systems**: Several strategies (e.g. membrane filtration, precipitation, solvent extraction, desalting, size exclusion chromatography) will be combined and optimised to remove residual components from culture supernatants of both synthetic media and buffer system. GABA in cleaned supernatants will be separated by using chromatography approaches, combining different resins and elution parameters (A2.1).

A3) **Microencapsulation of GABA-producer strain and purified GABA**: Different food-grade biopolymers/hydrocolloids, lipids and/or proteins will be combined and used as coating agents to produce spry-dried microencapsulated GABA-producing cells or purified GABA (A3.1). Encapsulation efficiency will be evaluated; a cost analysis will be carried out to identify the best protocol of microencapsulation (A3.2).

A4) **Food functionalization with GABA-producer strain or purified GABA**: Fermented milks and/or fruit juices will be used as model foods, and will be functionalized through an *in-situ* bioconversion using the microencapsulated producer strain (A4.1) or purified GABA. The chemical-physical and sensory properties of GABA-enhanced foods will be evaluated over-time to verify the effect of GABA supplementation (A4.2).

A5) **Bibliographic research, Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications

Table 1: *Gantt diagram for this PhD thesis project.*

		Year (1)						Year (2)						Year (3)					
Activity / Months		2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1	***Optimization of biomass and GABA production***																		
	1) Cultivations in bioreactor																		
	2) Analyses of biomass and GABA																		
A2	***Separation and purification of GABA***																		
	1) Chromatography techniques																		
A3	***Microencapsulation (ME) of GABA-producer strain and purified***																		
	1) Optimisation of ME protocol																		
	2) Evaluation of ME efficiency																		
A4	***Food functionalization with GABA***																		
	1) Development of GABA-enhanced foods and beverages																		
	2) Chemical-physical and sensory analysis																		
A5	***Bibliographic research, thesis and paper preparation***																		

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Strategie innovative di contrasto all'instabilità fenolica e alla perdita di longevità dei vini rossi

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This PhD thesis research project is aimed at investigating the physical-chemical phenomena involved in red wine instability and in the loss of longevity due to changes in the phenolic composition of grapes and wines linked to climate change. Innovative and sustainable oenological strategies to contrast the phenolic instability considering the specific composition of most diffused national and international grape varieties will be explored and pointed out.

Strategie innovative di contrasto all'instabilità fenolica e alla perdita di longevità dei vini rossi

Questo progetto di tesi di dottorato avrà come obiettivo generale lo studio dei fenomeni chimico-fisici responsabili dell'instabilità e della perdita di longevità dei vini rossi dovuta agli squilibri nella composizione fenolica dei vini determinati dal cambiamento climatico. Saranno inoltre messe a punto strategie enologiche innovative e sostenibili da impiegare in vinificazione per risolvere tali criticità tenendo conto delle specificità compositive delle principali varietà di uva da vino nazionali e internazionali.

1. State-of-the-Art

In recent years climate changes and global warm caused an imbalance in the phenylpropanoid way resulting in significant changes in phenolic composition of grapes and wines. Some of these effects can be detrimental for wine quality. As an example, for specific grape cultivars such as the Sangiovese variety (*Vitis vinifera* L.), an excessive synthesis of flavonols, especially quercetin occurred. During winemaking quercetin is transferred from grapes to wine but, when the amount of quercetin exceeds the solubility value, the formation of undesirable deposits in bottled red wines occurs. These deposits determine great economical loss for wine producers because Sangiovese is the most widespread grape cultivar in Italy (about 85,000 ha) (J. F. Vouillamoz *et al*., 2007).This happens because Quercetin (Q) is a phenolic compound belonging to the class of flavonols which are in berry skins as glycosides and are involved in UV screening (I. Hermosín-Gutiérrez *et al*., 2011) and their biosynthesis is greatly influenced by exposure to sunlight. Unfortunately, quercetin solubility in wine is affected by numerous factors and different values were described in literature (Table 1). In this scenario it is necessary to better understand which factors are affecting the solubility of Q in red wine and to find a sustainable strategy to limit the precipitation of Q in wines.

Table 1 Values of solubility of the quercetin in water and red wine described in literature.

Concentration (mg/L)	Matrix	References
2,63 mg/L	Water at 20 °C	*Chebil et al.,2007*
Increases with ethanol content	Hydro-alcoholic solution	*Srinivas et al.,2010*
125 mg/L	Musts and Wine	*Price et al., 2009*
5 mg/L	Wine	*Boulton., 2001*
15 mg/L	Sangiovese wines	*Gorelli., 2020*
3 mg/L	Hydro-alcoholic solution/red wines	*Gambuti et al., 2020*

Another important consequence of climate change is the loss of anthocyanins and the discrepancy between technological and phenolic ripening kinetics. This determines a loss of colloidal stability of red wines with the formation and precipitation of high molecular pigmented structures which determine a great loss of longevity of wines with consequent great economical loss for wine sector.
Although it is known that these phenomena are linked to changes in phenolic composition of wines and the necessity to find technological tools to afford these criticisms, given the great complexity of wine solution, most of these phenomena are still not well understood.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities

according to the Gantt diagram given in Table 2:

A1) **Determine a method of analysis for the quantification of flavonols and colloidal stability of red wines.**
1) To individuate sample extraction and quantification procedures.
2) Validation of methods of analysis.

A2) **To evaluate factors affecting solubility and precipitation of Q in red wines.**
1) Copigmentation and phenolic composition.
2) Temperature and time.

A3) **To evaluate factors affecting the hydrolysis kinetics of flavonol and anthocyanin glycosides in red wines**
1) pH and acidity.
2) Enzymes.

A4) **Individuate oenological strategies useful to contrast phenolic instability of wines by means of laboratory trials.**
1) Glycosidasic enzymes.
2) Adsorbent materials.
3) Addition of sustainable protective colloids.

A5) **Scale-up** optimal strategies individuated by means of laboratory trials in (A4) in an industrial scale.

A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Determine a Method of analysis for quantification of flavonolos and colloidal stability of red wine***																								
	1) Sample extraction and quantification procedure																								
	2) Validation of methods of analysis																								
A2)	***To evaluate factors affecting solubility and precipitation of Q in red wines.***																								
	1) Copigmentation and phenolic composition.																								
	2)Temperature and time. Module																								
A3)	***To evaluate factors affecting the hydrolysis kinetics of flavonols and anthocyanins glycosides in red wines.***																								
	1) pH and Acidity																								
	2)Enzymes																								
A4)	***Individuate oenological strategies useful to contrast phenolic instability of wines by means of laboratory trials***																								
	1) *Glycosidasic enzymes.*																								
	2) *Adsorbent materials*																								
	3) Addition of sustainable protective colloids																								
A5	***Scaling-up***																								
A5)	***Thesis and Paper Preparation***																								

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Applications of enzymes and membrane processes in peptide production

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This PhD project aims to develop an immobilized enzyme bioreactor coupled to an electrodialysis with ultrafiltration membranes system for the production and fractionation of peptides from the hydrolysis of whey proteins.

Applicazioni di enzimi e processi a membrana nella produzione di peptidi

Questo progetto di dottorato mira allo sviluppo di un bioreattore ad enzima immobilizzato accoppiato ad un sistema di elettrodialisi con membrane di ultrafiltrazione per la produzione e il frazionamento di peptidi mediante idrolisi di sieroproteine.

1. State-of-the-Art

Proteins, as a result of the cleavage of peptide bonds, are broken down into peptides of different sizes and free amino acids. This degradation, termed hydrolysis, can be carried out by enzymes (proteolysis), acids or alkali. Acid and alkaline hydrolysis tends to be a difficult process to control and yields products with reduced nutritional qualities. Enzymatic hydrolysis is developed under mild conditions of pH (6-8) and temperature (40-60° C), avoiding the extremes usually required for chemical and physical treatments and minimizing side reactions (Clemente, 2000). Proteolysis is becoming a method of choice in the food and pharmaceutical industries. Peptides derived from proteolysis can be used to develop nutraceuticals or functional foods with improved biological functions such as antioxidant, antihypertensive, antimicrobial activities, among others (García *et al.*, 2022). In addition to the classical hydrolysis using free enzymes, in recent years, research has been oriented towards the use of enzymes immobilized on various supports which has some advantages such as increased enzyme stability, enzyme re-cycle, use of high enzyme to substrate ratios. However, for the latter, it is crucial to select the appropriate immobilization supports having a high superficial density of reactive groups, as well as suitable immobilization conditions, such as reaction time, pH, temperature, buffers, and inhibitors or protein protectors, to enhance the enzyme–support reaction (Bortone *et al.*, 2012).
A protein hydrolysate contains many peptides of which only some have a biological activity. Therefore, there is a need to identify and characterize the latter, which begins with their fractionation. The main drawback of peptide fractionation is that most of the peptides share very similar physicochemical characteristics, therefore, only a separation technology able to distinguish between subtle differences in charge, size, solubility or hydropathicity results of utility (Fernández *et al.*, 2013). Over the years, various peptide fractionation processes have been studied such as chromatographic processes (Wafaa *et al.*, 2022) and membrane technologies. The latter is a low-cost, environmentally friendly technology, which works under mild operating conditions and leave the substrate nutritional properties almost intact. Among membrane processes, nanofiltration is considered as especially appropriate for peptide separation, due to the molecular weight cut-off used and the importance of charge effects. The combination of nanofiltration and ultrafiltration has also been used (Arrutia *et al.*, 2016). On the other hand, electrodialysis with ultrafiltration membranes which is an electromembrane process is gaining increasing interest for the recovery of charged molecules, especially bioactive peptides as part of sustainable strategies (Geoffroy *et al.*, 2022). So far, the production of bioactive peptides has mostly been operated in batch mode with free enzyme. This presents some disadvantages such as the higher energy consumption due to the long processing time, the product accumulation that acts as an inhibitor of the hydrolysis (Sousa *et al.*, 2004) and the fact that the enzyme can be only used once. To overcome these problems, this PhD project will be directed to develop a more viable system based on an immobilized enzyme bioreactor to be coupled with an electrodialysis with ultrafiltration membranes system to continuously produce bioactive peptides from whey proteins with the advantages of lowering the number of unit operations, the amount of enzyme used and the energy consumption.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Immobilization studies.** Screening of the scientific literature for available commercial enzymes for whey protein hydrolysis (A1.1). Choice of the enzyme/enzymes to use based on several aspects (degree of hydrolysis, potential bioactivity of peptides released) (A1.2). Development of the immobilization process/processes (A1.3). Characterization of the obtained immobilized biocatalysts (immobilization yield, immobilized activity, stability) (A1.4).

A2) **Bioreactor development.** Set up of an immobilized enzyme bioreactor (A2.1). Study of operating conditions and reactor configuration on hydrolysis degree (A 2.2).

A3) **Development and testing of electrodialysis system.** Identification of the ED with ultrafiltration membranes configuration and setting up of the system (A3.1). Separation tests with protein hydrolysate produced by the enzymatic reactor (A3.2). Development of the combined reactor with the electrodialysis system and separation tests (A3.3). Characterization of separated fractions of peptides (A3.4).

A4) **Design of experiments, development of empirical and/or mechanistic models of the system and data analysis.** Design of experiments (A4.1). Modeling of the enzyme kinetics for free and immobilized systems (A4.2). Basic modeling of the electrodialysis system (A4.3). Data analysis through univariate and multivariate techniques (A4.4).

A5) **Writing and Editing** of the PhD thesis, scientific papers, oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	30
A1)	***Immobilization studies***																									
	1) Selection of appropriate enzymes																									
	2) Studies of enzymes characteristics																									
	3) Immobilization process development																									
	4) Biocatalyst characterization																									
A2)	***Bioreactor development***																									
	1) Set up of immobilized enzyme bioreactor																									
	2) Study of hydrolysis degree																									
A3)	***Development and testing of electrodialysis system***																									
	1) Set up of the electrodialysis system																									
	2) Separation tests of protein hydrolysis																									
	3) Separation with the combined reactor with ultrafiltration system																									
	4) Characterization of separated fractions																									
A4)	***Development of empirical and/or mechanistic models of the system and data analysis***																									
	1) Design of experiments																									
	2) Modeling of the enzyme kinetics																									
	3) Modeling of the ED system																									
	4) Data analysis																									
A5)	***Thesis and Paper Preparation***																									

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Fermented sausage microbiome: investigation, storage and exploitation

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The aim of this doctoral research project is to study the microbiome of spontaneously fermented sausages and to define protocols for its conservation and propagation for subsequent exploitation in different ecosystems, using a multi-omics approach.

Microbioma di salami fermentati: studio, conservazione ed utilizzo

L'obiettivo di questo progetto di dottorato è studiare il microbioma di salumi a fermentazione spontanea e definire protocolli per la conservazione e propagazione del microbioma per un successivo riutilizzo in diversi ecosistemi, utilizzando approcci multi-omici.

1.State-of-the-Art

The food fermentation process was an ancient form of food preservation technology. Nevertheless, the technology has evolved beyond food preservation to become a tool for creating desirable organoleptic, nutritional, and functional attributes in food products, and has become a cultural and traditional norm within communities. The particular characteristics of typical fermented foods from each region are linked to the use of local ingredients and specific production techniques. In particular, the qualitative characteristics of spontaneously fermented sausages depend on the quality of ingredients and raw materials, and on microbial composition involved in specific processing and ripening conditions (Aquilanti et al., 2007).
Meat fermentation is driven by a complex succession of microbial consortia as weel as by biochemical transformations able to produce a variety of metabolites responsible for flavors, odors, and texture (Srinivas et al., 2022). In modern sausage production, the use of starter cultures is increasing to guarantee safety and standardize the properties of the final product (Cocolin et al., 2001). However, commercial starters, mainly composed by lactic acid bacteria and coagulase-negative cocci, reduce the peculiar organoleptic characteristics of spontaneously fermented sausages and lead to losses the typicity (Daga et al., 2021, Franciosa 2022). Besides, according to the FAO report "The State of the World's Biodiversity for Food and Agriculture", the biodiversity present in our ecosystems is in decline worldwide (FAO,199). Consequently, knowledge and conservation of the microbial biodiversity of spontaneously fermented sausages play an important role in protecting typical national products in order to maintaining biodiversity and sustainability.
Therefore, this PhD thesis project will be to study of the microbiome of Italian fermented sausages, through:
-characterization of community structure, diversity, activity and interactions in their natural environments;
-optimization of conditions and protocols for long-term preservation and propagation of microbiomes;
-identification of analytical methods to follow the structure of the microbiomes during the storage and the propagation.

2.PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram display in Table 2:

A1) **Definition of Standard Operation Procedures (SOPs)** for the sampling (A1.1) and DNA extraction from fermented sausages (A1.2)
A2) **Study the microbiome of different typical spontaneously fermented sausages** using microbiological (A2.1), metabolomic using GC-MS instrument (A2.2) and metagenomic (Shotgun sequencing) analysis (A2.3).
A3) **Optimization of conditions and protocols** for long-term preservation and propagation of microbiomes using different preservation matrices (pellet, meat, first 10-fold serial dilution) (A3.1) and propagation matrices (Nutrient Broth, Brain heart infusion, meat juice) (A3.2)**.**
A4) **Identification of analytical methods** to follow the quality of microbiomes during storage and propagation using microbiological (A4.1), metabolomic (A4.2) and metataxonomic (16S sequencing) analysis (A4.3).
A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1. Gantt diagram for this PhD thesis project.

Activity months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***SOPs***																								
	1)SOPs definition																								
	2) SOPs Validation																								
A2)	***Study of the microbial ecology***																								
	1) Microbial and sensory analysis																								
	2) Metabolomic analysis																								
	3) Metagenomic analysis																								
A3)	***Protocols optimization***																								
	1) Microbiome preservation protocols																								
	2) Microbiome propagation protocols																								
A4)	***Quality control of microbiomes***																								
	1) Microbial analysis																								
	2) Metabolomic analysis																								
	3) Metataxonomic analysis																								
A5)	***Thesis and Paper Preparation***																								

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Exploiting fermented foods microbiome to improve food quality and human health

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This PhD research project is aimed at investigating the microbial biodiversity in fermented foods and its potential implications for human health. The complex microbial communities of fermented foods will be investigated using a shotgun metagenomic approach, focusing on the microbiome functional potential, and highlighting the metabolic pathways involved in health-promoting activities. Finally, a long-term human intervention trial with milk kefir will be carried out to test *in vivo* the effects on gut microbiome and human health.

Utilizzo del microbioma degli alimenti fermentati per migliorare la qualità del cibo e la salute umana

Questo progetto di ricerca di dottorato mira a studiare la biodiversità microbica negli alimenti fermentati e le sue potenziali implicazioni per la salute umana. Le complesse comunità microbiche degli alimenti fermentati saranno studiate utilizzando un approccio di metagenomica shotgun, focalizzandosi sul potenziale funzionale del microbioma ed evidenziando i percorsi metabolici coinvolti nelle attività di promozione della salute. Infine, sarà condotto uno studio di intervento a lungo termine sull'uomo con kefir di latte per verificarne *in vivo* gli effetti sul microbioma intestinale e sulla salute umana.

1. State-of-the-Art

Fermented foods (FFs) have always been part of human cultures, including culinary traditions and health practices. These foods undergo microbial fermentation, a process in which complex microbial communities transform the raw ingredients into flavourful, preserved, and nutritionally rich products. Beyond these attributes, FFs have been associated with potential health benefits, ranging from improved digestion and nutrient absorption to enhanced immune function (Marco et al., 2017). The health benefits attributed to FFs stem from various factors. Microorganisms produce an array of bioactive compounds during fermentation, including organic acids, antimicrobial peptides, and vitamins, many of which have physiological effects on the human body. Furthermore, fermentation increases the bioavailability of nutrients, making them easier to be absorbed (Cani, 2018; Sharma et al., 2020). Numerous FFs have been tested on humans, investigating their potential effects and benefits. In Table 1 main results from intervention studies are summarised. Understanding the intricate relationship between FFs and human health has been greatly advanced by the application of shotgun metagenomic sequencing, which allows for a comprehensive analysis of the genetic material present in complex microbial communities, enabling the identification of microbial strains and their associated functional genes (Quince et al., 2017). Through bioinformatic analysis, reconstruction of metagenome-assembled genomes can assess metabolic capabilities of FFs microbiome, unravelling the microbial diversity and functional dynamics, offering a deeper understanding of the potential health-promoting properties, including immune-modulating functions, metabolic processes, and mental wellness (De Filippis et al., 2020).

Table 1 Main FFs used for intervention studies exploring effects in human health.

Diet or fermented food matrix	Study design	Variations in the gut microbiome	Health outcome targeted	Health outcome achieved
High-FFs diet	RCT	↑ *Ruminococcaceae* and *Streptococcaceae* ↓ *Lachnospira*	Inflammation	Yes
Grana Padano	Double blind placebo-controlled	Not detected	Hypertension	Yes
Kefir	RCT	↑ *Lactobacillus* and *Bifidobacterium* spp.	Metabolic syndrome	Yes
Fermented milk	Cross-over trial	↑ *Bacteroides ovatus, Lachnospira and Ruminococcus*	IBS and obesity	No
Kimchi	RCT	↑ *Prevotella* and *Bacteroides* ↓ *Blautia*	Metabolic syndrome	Yes

Yogurt	Before/after trial	↑ LAB and *Clostridium perfringens* ↓ *Bacteroides*	None	None

FFs: fermented foods; RCT: randomised control trial; IBS: irritable bowel syndrome: LAB: lactic acid bacteria.

While shotgun metagenomic sequencing has improved our understanding of FFs and their impact on human health, challenges remain. Large-scale studies and well-designed intervention trials are needed to establish causal relationships between FFs consumption, gut microbial community changes, and health outcomes (Johnson et al., 2020). This PhD project develops within the framework of the EU project DOMINO (GA 101060218). Within this project, a database of FFs metagenomes will be developed, aiming to map the complex microbial communities present in different types of FFs. The database will be screened to identify microbial markers (species, strains and/or genes) potentially associated with positive health outcomes. Finally, a randomised controlled trial in subjects with metabolic syndrome will be developed to highlight the effect of FFs on human health and the FFs-gut microbiome axis.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) **Sampling of FFs and beverages** (e.g., cheeses and dairy products, kombucha, fermented vegetables), in order to expand the knowledge on FFs microbiome (A1.1). The analysis of molecular compounds (Volatile Organic Compounds, antioxidants) will be also performed (A1.2).

A2) **Shotgun metagenomic sequencing** will be carried out to investigate microbial communities in different FFs. Thus, microbial DNA will be extracted, and metagenomic libraries prepared and sequenced (A2.1). Then, metagenome bioinformatic analysis will reveal FFs microbial strains and predict metabolic pathways related to them (A2.2).

A3) **A database of FFs microbiome** will be obtained through the post-processing of metagenomic data (A3.1) and a large-data statistical analysis (A3.2). Microbial genes related to potential health effects will be identified.

A4) **FFs human trial** to validate the health effect of FFs, as well as the effect on the gut microbiome and the potential transfer of microbial strains.

A5) **Writing and editing** of the PhD thesis, scientific papers and oral/poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Sampling of FFs and beverages***																								
	1) FFs sampling																								
	2) Metabolomics																								
A2)	***Shotgun metagenomic sequencing***																								
	1) DNA extraction and sequencing																								
	2) Metagenome bioinformatic analysis																								
A3)	***FFs database construction***																								
	1) Post-processing of metagenomic data																								
	2) Big-data statistical analysis																								
A4)	***Trial/intervention with FFs***																								
A4)	***Thesis and paper preparation***																								

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Technological strategies for by-products valorisation and innovative food developments

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This PhD project aims to propose technological strategies to improve the reuse and recycle of by-products as alternative sources of nutrients. Food loss and waste (FLW) represent a good candidate for the requirements of a better sustainable food system. Their transformation and reuse for innovative food products development would contribute to reduce the current environmental impact. Some FLWs will be selected based on their nutritional and functional characteristics, also evaluating local by-products. They will be utilised as alternative ingredients for designing and developing innovative end-products with high nutritional and sensorial properties, also employing emerging technologies, such as 3D food printing.

Strategie tecnologiche per la valorizzazione di scarti alimentari e lo sviluppo di prodotti innovativi

Questo progetto di dottorato propone di incentivare, attraverso strategie tecnologiche, il recupero e il reimpiego di scarti e sottoprodotti alimentari come fonti alternative di nutrienti, rappresentando una strada percorribile per rispondere alla richiesta di una maggiore sostenibilità del sistema alimentare. La loro trasformazione e riutilizzo contribuirebbero a ridurre l'impatto ambientale dell'attuale modello produttivo. Alcuni scarti e sottoprodotti saranno selezionati per il loro contenuto nutrizionale e caratteristiche funzionali, anche considerando sottoprodotti locali, i quali saranno utilizzati come ingredienti innovativi per progettare e sviluppare prodotti finiti, ad elevato valore nutrizionale e apprezzabile qualità sensoriale, anche impiegando tecnologie emergenti come la stampa 3D.

1. State of the art

Global food waste has been estimated at around 1,3 billion tons a year. This matter has been reported as a social, environmental, and economic challenge by FAO (2019). Indeed, such a waste of food represents a paradox if we consider that, in the world, around 800 million are malnourished and this data is destined to increase up to 100% within 2050. The increasing food demand is compelling the agro-food industry to exert unsustainable pressure on natural resources, such as soil, air, water, and biodiversity. The global incidence of agriculture and farms is estimated to account for up to 30% of gas emissions and up to 70% for use of water. In this context, and considering the growth of the population, using resources more efficiently and reducing gas emitted will be paramount in meeting increasing demand in a sustainable way. Moreover, among the main nutritional deficiencies registered, there are proteins. In some parts of the world, it is arduous to supply protein sources, such as meat and fish, considering the production cost and not availability of farms and sea; so as for some population targets, like low-income and elderly people (FAO, 2018). For these reasons it is necessary to explore the possibility of exploiting alternative vegetable protein sources, which could represent a cheaper alternative, especially if derived from waste and by-products. In this scenario, new food production technologies need to be investigated and optimised, implementing the circular economy. Currently, the most utilised recycling technologies are based on the extraction of functional compounds, such as phenols, and proteins (Munialo et. al., 2022). These methods require the application of chemical reagents or microwaves, that imply high costs and energy expenditure and do not represent an effectively sustainable solution. Consequently, more suitable technologies need to be investigated and suggested, following an eco-friendly perspective. In view of the previously mentioned challenges, the research of new sources of nutrients, especially those with a high protein content, sustainable and healthy, is among the main priorities of European strategies (Horizon Europe, 2021-2027). For these reasons, in recent years, researchers have focused attention on by-products and food waste as nutrient sources. In Table 1 are reported some examples of vegetable wastes and derived innovative products, selected based on their functional and nutritional properties, local availability, sustainability, and amount of waste produced.

Table 1 *Some applications of selected by-products and waste in food products*

Vegetables waste/ byproduct	Main nutrients and functional molecules	Developed products	References
Almond skins	Phenolic compounds, fibres	Bread	Gaglio et al., 2023
Artichoke (stems, leaves, external bracts)	Fibres (inulin), minerals,	Fortified spreadable cheese	Soares Mateus et al., 2023

	phenolic compounds		
Okara	Proteins, fibres	Biscuits	Shan Lee et al., 2020
Canola (oil extraction residual)	Proteins	Meat analogues, bakery products, snacks	Chmielewska et al., 2020

2. PhD Thesis Objectives and Milestones

Considering the aforementioned challenges, the present Phd project has the main objective to study and employ alternative sources of proteins and other nutrients, also deriving from waste of the food industry, in order to create innovative food products with high nutritional and healthy value, with sensory properties widely appreciated by consumers. It can be divided into the following specific objectives and activities according to the Gantt diagram given in Table 2:

OS1) Development of **by-product-based novel food ingredients** in different forms (e.g., powder, viscous liquids, paste, etc.) with nutritional and technological properties tailored for the targeted end-products.

T1.1) Data analysis from different available databases to identify the most important technological properties affecting the development of targeted food products (e.g., bread, biscuits, fruit juice, etc.).

T1.2) Novel ingredients development in form of powders or viscous liquid: by testing and optimising technological methodologies - e.g., different dehydration techniques, grinding methods and conditions, homogenization, and stabilisation processes.

T1.3) Technological, nutritional, and sensorial characterization of the obtained ingredients and their mixing tailored for the target food products consumer's demands.

OS2) **Design, development, and test of novel nutritionally enhanced food products by** using traditional and innovative techniques.

T.2.1 - Traditional techniques: study of mixing, leavening, and cooking steps with enriched bakery products.

T.2.2 - Innovative techniques: use of the 3D printing technology, optimization of digital model design and printing parameters.

T.2.3 - Nutritional and sensory analysis.

OS3) **Writing and Editing** of the PhD thesis, scientific papers, oral and poster communications.

Table 2 *Gantt diagram for this PhD thesis project.*

Activities / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
OS1)	**Ingredients realisation**																								
	T1.1) Data analysis																								
	T1.2) Ingredients development																								
	T1.3) Qualities determination																								
OS2)	**Products development**																								
	T2.1) Traditional techniques																								
	T2.2) Innovative techniques																								
	T2.3) Qualities analysis																								
OS3)	**Thesis and Papers writing**																								

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Impact of vegetable proteins on gut microbiome modulation and health promotion

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This PhD thesis research project is aimed to investigate the impacts of plant-based diet, with an emphasis on legumes consumption, on gut microbiome and health outcomes in healthy individuals. The main focus is to understand how replacing animal-derived with plant-based proteins may affect the composition and potential functionality of the gut microbiome, potentially influencing host health, inflammatory response, and immune function. By demonstrating the benefits of this dietary pattern on gut health and overall well-being, this study has the potential to bring significant innovation to the plant-based food industry by adding value and differentiation to new food and dietary proposal.

Impatto delle proteine vegetali sulla modulazione del microbiota intestinale e sulla promozione della salute

Questo progetto di tesi di dottorato mira ad indagare gli effetti di una dieta a base vegetale, con un'enfasi sul consumo di legumi, sul microbiota intestinale e sulla salute di individui sani. L'obiettivo principale è comprendere come la sostituzione di proteine animali con quelle vegetali influenzi la composizione e la potenziale funzionalità del microbiota intestinale, incidendo sulla salute dell'ospite, sull'infiammazione e sulla funzione immunitaria. Dimostrando i benefici di questo regime alimentare sulla salute intestinale e il benessere generale, questo studio ha il potenziale per apportare un'innovazione significativa al settore dei prodotti vegetali, aggiungendo valore e differenziazione alle nuove proposte alimentari.

1. State-of-the-Art

The gut microbiome, the complex community of microorganisms residing in our gastrointestinal tract, has significant influence on crucial human functions such as immunomodulation, behaviour, dietary nutrient and drug metabolism. The composition of this intricate community can be heavily influenced by long-term dietary habits (De Filippis et al. 2018). In recent years, plant-based diets (PBDs), characterized by a high intake of fruits, vegetables, legumes, and whole grains, and limited or absent consumption of animal-derived products, have gained global traction due to their benefits for individuals' health and the environment sustainability. Research suggests that long-term PBDs notably modify the gut microbiome, fostering fiber-degrading bacteria and enhancing beneficial short-chain fatty acid production (Sidhu et al. 2023). Conversely, diets high in animal-based foods tend to promote bacteria specialized in fat/protein metabolism, thus resulting in an increase in potentially harmful microbial by-products (David et al. 2014).
Table 1 outlines the main dietary patterns along with their sources of protein ordered by frequency of use and highlights how these dietary patterns have distinct influences on gut microbiota composition.

Table 1 Influences of dietary patterns on gut microbiota composition.

Dietary pattern	Source of proteins (ranked by frequency of use)	Effects on gut microbiota composition
Vegan / Vegetarian diet	Legumes, grains, nuts, seeds, plant-based meat alternatives	↑ *Faecalibacterium prausnitzii, Bacteroides, Prevotella, Bacteroides thetaiotaomicron, Bacteroidetes, K. pneumoniae, Clostridium clostridioforme* ↓ *Bifidobacteria, Clostridium cluster XIV, Bilophila*
Mediterranean diet	Dairy products, legumes, grains, nuts, seeds, fish, seafood, poultry, eggs, meat	↑ *Bifidobacteria, Lactobacillus, Lachnospiraceae, Bacteroidetes* ↓ *Clostridium, Enterobacteria*
Western diet	Meat, diary products, eggs, processed meats, poultry, fish, legumes, grains	↑ *Ruminococcus torques, Enterobacteria, Bilophila, Alistipes, Bacteroides, Akkermansia* ↓ *Bifidobacteria, Roseburia, Eubacterium rectale, Ruminococcus bromii, Lactobacillus, Prevotella*

↑: Increase in the abundance; ↓: decrease in the abundance.

The protein source is a key difference between plant and animal-based diets, and it can significantly impact gut microbiota composition (Christudas et al. 2020). For instance, animal proteins may increase bile-tolerant anaerobic bacteria, while plant proteins (e.g., pea proteins) can increase gut-commensal bacteria and decrease pathogenic ones (Rinninella et al. 2019). In PBDs, legumes provide a protein source with a comprehensive amino acid profile similar to animal proteins when combined with other vegetable proteins from cereals. Moreover, research has highlighted their role in lowering heart disease risk and improving various health biomarkers related to lipid and glucose metabolism and brain function (Martini et al. 2021). However, gaps remain in our understanding of the physiological impacts of legumes, particularly during a shift from a meat-rich to a plant-based, legume-rich diet. The interactions of this food with gut microbiome and the bioavailability of their vegetable nutrients are underexplored in vivo. Thus, this PhD thesis aims to explore the impact of a plant-based diet, that substitute animal-origin with plant-based proteins from legumes, on the gut microbiome of individuals prone to lifestyle-induced cardiovascular diseases. The research will investigate the interplay between diet, bioactive plant compounds, and the gut microbiome, and how these factors influence health outcomes.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) **Randomized controlled trial (RCT)** targeting high meat eaters with low physical activity (A1.1) will span over a period of 10 weeks. This includes a 2-week run-in and 8-week intervention period with regular anthropometric measurements (A1.2). Dietary and activity level questionnaires (A1.3) will monitor the compliance.

A2) **Biomarker collection and analysis** will be divided into two parts. The first one includes a metabolome and health marker analysis (A2.1) evaluating blood markers related to metabolism, inflammation, brain function, and oxidative stress. The levels of urinary, blood and fecal polyphenols and microbial metabolites associated with positive or negative effects (e.g., urolithins, equol, Trimethylamine N-oxide (TMAO)) will be also assessed. The second part will focus on gut microbiome analysis and data integrations (A2.2).

A3) **In-vitro SHIME (Simulator of the Human Intestinal Microbial Ecosystem) experiment** will be divided into two steps. Firstly, the fecal donor (A3.1) will be selected among the study participants. Secondly, the shotgun metagenomics analysis (A3.2) at different treatment times.

A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Randomized Controlled Trial***																								
	1) Subject recruitment																								
	2) RCT phases																								
	3) Dietary and activity levels assessment																								
A2)	**Biomarker collection and analysis**																								
	1) Metabolome and health marker analysis																								
	2) Microbiome analysis and data integration																								
A3)	***SHIME experiment***																								
	1) Fecal inoculum selection																								
	2) Shotgun metagenomic analysis																								
A4)	***Thesis and Paper Preparation***																								

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Smart solution for crop production exposed to biotic and abiotic stresses

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This PhD thesis research project is aimed at testing the use of *Streptomyces* as biostimulants, first in the growth chamber and then in the greenhouse, on plants under water stress conditions to investigate their mode of action, through -omics approaches and non-destructive tools, which assess the health status of the plant, in order to obtain a product that can be applied in the open field and that guarantees production even in arid climates.

Nuove soluzioni per la produzione di colture esposte a stress biotici e abiotici

Questo progetto di tesi di dottorato mira a testare l'utilizzo degli streptomiceti come biostimolanti, prima in camera di crescita e poi in serra, su piante in condizioni di stress idrico a investigarne il modo di azione, tramite approcci -omici e strumenti non distruttivi, che valutano lo stato di salute della pianta, al fine di ottenere un prodotto che possa essere applicato in campo aperto e che garantisca la produzione anche in climi aridi.

1. State-of-the-Art

Global agriculture is facing numerous challenges due to increasing demand for food and plant-based ecosystem services, driven by a predicted population growth from 7.7 billion to 9.7 billion by 2050. This necessitates sustainable agricultural practices to meet future food production goals (Camaille et al., 2021). Climate change, particularly the occurrence of drought, has a detrimental impact on crop productivity and is expected to worsen due to reduced soil water levels caused by increased temperatures (Camaille et al., 2021). Drought is a major abiotic factor limiting global crop productivity (Fahad et al., 2017), making it a crucial target of plant research. The ultimate objective is to develop crop plants with improved water use efficiency to minimize drought-induced yield losses and mitigate the threat of food scarcity (Mishra et al., 2012). Drought stress tolerance not only improves productivity on currently cultivated land but also allows the exploitation of cultivable land with limited water supplies. Plants employ various strategies to cope with drought, including maintaining high water status through efficient water absorption or reducing evo-transpiration. Drought-tolerant plants maintain turgor and metabolic activity even at low water potential through mechanisms like protoplasmic tolerance and synthesis of osmoprotectants (Mishra et al., 2012). Stomatal closure, mediated by abscisic acid (ABA), is triggered during drought stress to reduce water loss. However, closed stomata also decrease CO2 supply, leading to reduced photosynthetic activity and hindered plant growth. Drought-induced alterations in physiology, growth, metabolism, and production vary with the level of plant tolerance. Osmotic adjustment, a mechanism involving the accumulation of osmolytes like proline to stabilize and protect subcellular structures against oxidative damage. (Patanè et al., 2016). To ensure sustainable agriculture, the use of soil microorganisms, particularly plant growth-promoting rhizobacteria (PGPR), has gained attention. PGPRs not only promote plant growth but also have the potential to alleviate abiotic stress. Recent years have witnessed significant advancements in understanding the mechanisms of action of PGPRs. Thus, the use of PGPRs in agriculture is seen as a promising solution to enhance productivity and tolerance to both biotic and abiotic stresses. Endophytic actinobacteria have been shown to be able to adjust to both abiotic and biotic stresses (Mattei et al., 2022; Sathya et al., 2017; Yandigeri et al., 2012;), but no studies have been done on how they affect photosynthetic parameters (Passari et al., 2019).
Thus, this PhD project aims to study the plant-bacteria interaction under drought stress conditions using advanced non-destructive physiological methods and omics technologies. The goal is to correlate plant phenotype with molecular data to gain a comprehensive understanding of the interaction between the plant and PGPRs and how the plant physiological parameters can be related to this interaction. Moreover, by analysing the molecules and biosynthetic pathways involved in the system, *Streptomyces* can be exploited as a tool to ensure crop productivity in challenging environmental conditions.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:
WP1: Cultivation and selection of *Streptomyces* and seed coating of plant seeds. Plants are going to be grown in

growth chambers, to further run multi-omics analyses. Moreover, drought stress is going to be imposed during the green house tests and assessed by non-destructive tools. These tools are going to be used even for measuring *Streptomyces* effects *in-planta* by comparison of data collected from control plants (non-stressed).
In parallel, genomic analysis of *Streptomyces* and comparative genomic analysis can be used to build a valuable database to be used for the following omics studies.
WP2: Transcriptomics: Analysis of the expression of BCGs which can be involved the bacteria-plant interaction. At least 4 time points linked to the different stages of the interaction will be investigated. Identification similarities and differences at the transcriptome level during the interaction will be obtained by careful definition of key points to perform RNA studies at the appropriate key steps in the PGP interactions. Diversity in gene expression will be linked to possible specific interactions within the system.
Deliverables: Transcriptomic analysis of the crosstalk occurring in the system (*Streptomyces* – plant) and set of specific BCGs active in the PGPR process.
WP3: **Proteomics:** to investigate the relationship between the metabolic pathways and the natural products production. Proteomics gives information on differential pathways regulation, identifying major participants in natural product biosynthesis that can be exploited as targets for rational engineering, by comparing protein expression levels in different conditions. Deliverables: strain characterization by linking natural products to specific gene clusters.
WP4: Metabolomics analyses to investigate the response to biological stimuli of secondary metabolite producing strain. Measurement of the global levels of low molecular weight metabolites to obtain a metabolic comparison of the different biological samples without any chemical redundancy, to identify secondary metabolites from silent BCGs. Deliverables: fully characterized crosstalk in the PGP-plant interaction system.
WP 5,6,7: Field Trials and Hypothesis validation: The last working point will be the definition and the validation of the model in order to finally understand how the interactions occurs and which mechanisms are involved in the PGP interaction. Furthermore, the aim is to understand how *Streptomyces* stimulate the plant plant growth counteracting the drought stress by ensuring fruit production.
WP8: Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity		Months																																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1.	Strain preparation																																				
1.2	Seed coating with *Streptomyces*																																				
1.3	Plant growth in green house																																				
1.4	DNA Extraction and sequencing																																				
1.4	RNA extraction and optimization																																				
2.	**Transcriptomic analysis**																																				
2.1	Bioinformatics																																				
3.	**Proteomic analysis**																																				
3.1	bioinformatic																																				
4.	**Metabolomic analysis**																																				
4.1	bioinformatic																																				
5.	**Field trials**																																				
6.	**Data analysis**																																				
7.	**Mode of Action**																																				
8.	**Thesis and paper preparation**																																				

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Bacterial exploitation for heavy metal bioremediation and sequestration from wastewaters

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This PhD project has the purpose to study bacterial challenges to heavy metals (HMs) through the implementation of microbial resistance mechanisms for the decontamination of industrial and the subsequent recovery of HMs. It consists in an eco-friendly biotechnology which will permit to convert a waste product into a valuable one, since the metal-organic complexes can serve as commercial salts or catalysts. The final aim is to design a process that has a lower water footprint and fits in the concept of circular economy.

Biorisanamento e sequestro di metalli pesanti da acque reflue tramite l'utilizzo di batteri

Il presente progetto di dottorato si propone di studiare la capacità batterica di resistenti ai metalli pesanti tramite l'implementazione di meccanismi di resistenza microbica per la decontaminazione di acque reflue industriali e il successivo recupero di metalli pesanti. Si tratta di una biotecnologia eco-friendly che permetterà di convertire un prodotto di scarto in un prodotto di valore, poiché i complessi metallo-organici possono essere utilizzati come sali commerciali o catalizzatori. L'obiettivo finale è elaborare un processo che abbia una minore impronta idrica e si inserisca in un concetto di economia circolare.

1. State-of-the-Art

Over the last few decades, several industrial processes such as industrial welding, dyes and pigments manufacturing, electroplating processes, leather tanning, agricultural activities, and wood preservation, have been considered the major causes of HMs water pollution (Singh *et al.*, 2023). Such contaminants deteriorate water quality, with a negative impact on human health and ecosystems, due to their highly toxic, non-biodegradable and persistent nature (Nyika *et al*., 2022). The HMs that cause harmful impacts include, among others, chromium (Cr), copper (Cu) and nickel (Ni). These metals are responsible for reducing profitability and causing damage also to the agricultural systems (Yaashikaa *et al*., 2022). The United Nations world water development report 2021 claimed that around the world, 80% of all the industrial and municipal wastewater is directly released into the water ecosystems without any pre-treatment, leading to severe adverse effects on the environment (Jain *et al*., 2022). Among possible treatment strategies, the use of microorganisms represents a suitable solution (Jain *et al*., 2022). Their resistance mechanisms can be divided into: i) generic stress-related, and ii) HM-dependent ones. To the first one belongs the production of exopolymeric substances (EPS), outer membrane binding, precipitation as salts and sequestration by stress related peptides like glutathione. The second group includes periplasmatic accumulation and HM subsequent transformation to insoluble compounds by metallothionein-mediated transport, enzymatic activities of efflux pumps, and specific oxido-reduction mechanisms to transform metals into harmless substances. Moreover, HMs can undergo dissimilative processes when they are used as electron acceptors/donors for microbial metabolic purposes to produce energy (Nyika *et al*., 2022). However, there is still a gap of knowledge regarding the specific interactions metal-microorganism, in particular regarding interactions in multimetal contaminated environments. Furthermore, there are several challenges for large-scale industrial bioremediation application which feasibility needs to be explored. In addition, the optimal environmental conditions to enhance bacterial growth, the operating conditions and mechanisms of removal need to be critically evaluated (Jain *et al*., 2022). Mechanisms of wastewater decontamination exploit HM bindings with EPS bacterial surface (Singh *et al.*, 2023). EPS functional groups such as hydroxide (-OH), carboxylate (-COO), amino (-NH), and carbonyl (C = O), help to bind to HM cations, removing them from the contaminated solutions (Priyadarshanee *et al.,* 2021). A technique used to evidence the presence of EPS-producing cells is flow cytometry (FC), based on the specific interaction of EPS carbohydrates with lectins labelled with fluorophores (Hendrickson *et al.,* 2019). EPS is one of the significant components of biofilms which are clusters of microbial cells attached to a substratum and embedded within a matrix of EPS. The formation of bacterial biofilms can be employed in water treatments since the toxic metal ions can be entrapped within the biofilm. This technique has lately gained attention owing to its high microbial biomass density and immobilization capability, as well as more adhesive properties. The biological activation of adsorbing biomaterials (*i.e*. agro-wastes, biochar, activated carbon, lignite) with specific metal-removing bacterial strains can lead to the formation of microporous microcarriers activated with bacterial biofilms. These systems are characterized by selective HM extraction while providing cells with high resistance to environmental stress (Priyadarshanee *et al.,* 2021). The material used for adsorption should be easily available, non-toxic, and cost-effective (Manikandan *et al.,* 2023). In addition to the above-mentioned technology, bacteria

may form biofilm on the anode of microbial electrochemical systems (MES), considered innovative tools of wastewater treatment and bioremediation of HMs (Sivasankar *et al.,* 2019).
The focus of the present research will be to characterize HM resistant bacterial strains to provide insights for their possible exploitation in metal removal and recovery from industrial wastewaters. The present research will contribute to lower the industrial water footprint since the treated waters will re-enter the production cycle.

2. PhD Thesis Objectives and Milestones

The objective of the present PhD study is to test isolated bacterial strains for their feasibility to remove HMs from industrial wastewaters. According to the Gantt diagram given in Table 1 the activities of this PhD project will be subdivided as follows:

A1) ***In vitro essay*** will be set to test isolated bacterial strains in biofilm and planktonic experiments in the presence of HMs such as Ni(II), Cu (II) and Cr(VI) that will be determined by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) and spectrophotometric methods. Subsequently, trials on real HM contaminated electroplating wastewater will be conducted to assess adsorption and desorption kinetics and metals recovery from bacterial biomass.

A2) ***Characterization of mechanisms in HM/microbe interaction*** which will be assessed through i) EPS compositional characterization by nuclear magnetic resonance (NMR); ii) interaction between EPS components and specific lectins by FC; iii) definition of HMs detoxification pathways.

A3) ***Assessment of HM biofiltration unit*** through microporous microcarriers activated by bacterial biofilms monitored through lectins labelled with fluorophores.

A4) ***State of the art, dissemination, and thesis preparation*** by scientific literature revision, manuscript preparation and oral/poster communications to national/international conferences.

Table 1 Gantt diagram for this PhD thesis project.

Activity/Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***In vitro essay***																								
	1) Biofilm and planktonic experiments on Ni(II), Cu (II) and Cr(VI)																								
	2) Adsorption/desorption kinetics																								
	3) Metal recovery from biomass																								
	4) Trials on real electroplating wastewaters																								
A2)	***Characterization of mechanisms in HM/microbe interaction***																								
	1) ESP (NMR and flow cytometry)																								
	2) HMs detoxification pathways																								
A3)	***Assessment of HM biofiltration unit***																								
	1)Active biofilm on microporous microcarrier																								
	2) International internship																								
A4)	***State of art, dissemination, and thesis preparation***																								

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Intelligent sensors and laser spectroscopy for improving quality and prolonging the shelf life of food without chemical additives.

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The shelf life of a product is defined as the duration for which a product remains safe.
Today, there are several strategies to prolong it by using alternative systems to chemistry to promote a reduction in the use of food additives.
There are also many methods to monitoring, but they are almost exclusively almost exclusively destructive.
One solution to this problem could be to use non-invasive sensors and instrumentation allowing the monitoring of the product characteristics through non-destructive analysis methods.

Sensori intelligenti e spettroscopia laser per migliorare la qualità e prolungare la schelf-life degli alimenti senza additivi chimici.

La shelf life di un prodotto è definita come la durata per cui un prodotto rimane sicuro.
Oggi esistono diverse strategie per prolungarla, utilizzando sistemi alternativi alla chimica per promuovere una riduzione dell'uso di additivi alimentari.
Esistono anche molti metodi di monitoraggio, ma sono quasi esclusivamente di tipo distruttivo.
Una soluzione a questo problema potrebbe essere l'utilizzo di sensori e strumentazioni non invasive che consentano il monitoraggio delle caratteristiche del prodotto attraverso metodi di analisi non distruttivi.

1. State of the Art

The shelf-life is usually defined as the time during which a food product remains safe, in compliance with the label declaration of nutritional data, and retains the desired sensory, chemical, physical, and microbiological characteristics when stored under the recommended conditions (Institute of Food Science and Technology (Gran Bretaña)., 1993). Extending the shelf-life of a product allows it to preserve its organoleptic and nutritional quality as long as possible for the benefit of the consumer (Giménez et al., 2012) and reduce the incidence of waste.
The shelf-life of food products and beverage estimation has become increasingly important in recent years due to technological developments and the growth in consumers' interest in eating fresh, safe, and high-quality products. The shelf-life of the majority of food products is determined by changes in their sensory characteristics (Giménez et al., 2012; Hough, 2010).
In this context, sensory shelf-life estimation become an issue of continuous and extensive research about both the deteriorative mechanisms occurring in food systems and the development and application of methodologies for shelf-life estimation (Manzocco & Lagazio, 2009).
One way to improve shelf life, under a circular economy perspective, could be to use food by-products and their bioactive compounds to eliminate the chemical preservatives perceived as potentially harmful to health by an increasing number of consumers (Ghanbari et al., 2013; Hassoun & Emir Çoban, 2017).
By-products such as peel, pulp, husk, seeds, bagasse, barks, oil cake, etc. are readily available and constitute about 30–50% of the total food weight. The utility of by products can be evaluated by its composition and the cost of extraction of valuable compounds. Some by-products retain a percentage of bioactive compounds like flavonols, polyphenols, and tannins that may contribute to create an innovative packaging system with antioxidant and antimicrobical compounds (Bañón et al., 2007; Jiang et al., 2020; Jönsson & Martín, 2016).
One of the principal uses of such antioxidant and antimicrobial compounds could be the encapsulation by spray-drying, a popular technique thanks to its simplicity, affordability, and easiness in transportation and use of the powder form (Assadpour & Jafari, 2019; Vinceković et al., 2017).
In addition to the phenolic compounds, edible chitosan-based protective films could be used arthropods repellents to keep food unaffected for longer (Perwita et al., 2020).
In recent years, the need to monitor the parameters that affect the production and storage of food and to improve its quality and shelf-life, with sensors and/or laser spectroscopy, is increasingly developing. In particular, manysensors are already present "in line" in the production chains, but the vast majority of them use destructive. Such methods, do not allow a second sampling of the product and determine a waste of the same, since it is impossible to sell the sampled product.
The use of spectroscopy and sensors can reduce food waste caused by destructive analysis.
Spectroscopy in particular allows, through an emitter, a receiver, and a beam working in the NIR, to analyze the concentration of a given gas inside a container without opening it.
Some sensors, as those developed by INFN in the work Manzella (2022), Mercanti (2022), Vicidomini (2022) allow the reading of certain parameters without doing destructive analysis too.

2. PhD Thesis Objectives and Milestones

The main objective of this PhD project is to identify optimal methods and conditions for increasing the shelf-life of products (ex: wine, oil) without the addition of chemical additives. In particular:

A1) Sensors will be developed ad hoc for the monitoring of some stages of production and storage of the product.

A2) Attention will be given to the various stages of process and storage of the various products and conservation of the various products to identify, always by means of sensors, any criticality of the same (fermentation, kneading, leavening, bottling, bag in bag...) until you come to understand what may be the best conditions of packaging and product maintenance (humidity, controlled atmosphere...) that extend the shelf life without the added chemistry.

A3) There will be monitoring of the environmental conditions that will allow you to continuously follow the temporal trends of the pressures, temperatures and light conditions inside the bottles being aged. By crossing analytical results it will be possible to determine any differences in wines, and to bring them back to different evolutionary mechanisms.

A4) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 *Gantt diagram for this PhD thesis project.*

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Developed of TaylorMade sensors***																								
	1) Optimization of operative conditions																								
	2) Chemical and sensory analysis																								
A2)	***Optimization of stages of process and storage***																								
	1) Modification of the main chemical-physical parameters																								
	2) Shelf-life test																								
A3)	***Development of new products and packaging***																								
	1) Chemical-physical characterization																								
	2) Sensory characterization																								
	3) Evaluation of shelf-life of the product and of the optimum storage conditions																								
A4)	***PhD thesis editing***																								

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From research to business: Technology transfer models in the field of Food Science

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This PhD thesis project is aimed at the implementation of a model for the scale-up of food innovative solutions proposed by research, on a scale closer to the industrial one. Food prototypes developed at laboratory level (Technology Readiness Level, TRL 2-3), will be validated and designed at the industrial level (TRL 5-6). The project issues are in line with the mission 4 component 2 "M4C2" purpose of the National Recovery Plan and Resilience document, that provide to support investments in research and development, to promote innovations and their transfer to the food companies.

Dalla ricerca all'impresa: Modelli di trasferimento tecnologico in ambito Food Science

Questo progetto di tesi di dottorato mira alla realizzazione di un modello di scale-up industriale applicabile in ambito food science. In particolare, le soluzioni innovative proposte e sviluppate dalla ricerca, su scala laboratoriale (Technology Readiness Level, TRL 2-3), verranno convalidate in scala più vicina a quella industriale, (TRL 5-6), in linea con l'obiettivo M4C2 del documento Piano Nazionale Ripresa e Resilienza Italia. In quest'ultimo, infatti, la missione 4 e componente 2, prevedono di sostenere gli investimenti in ricerca e sviluppo, di promuovere l'innovazione e la diffusione delle tecnologie, di rafforzare le competenze, favorendo la transizione verso una economia basata sulla conoscenza.

1. State-of-the-Art

Food firms are one the most important manufacturing industries of the world. However, the food manufacturing and technology sector has typically been regarded as "low tech", lacking in innovative capacity. Despite this aspect, food firms are increasingly open to innovation practices, driven mostly by the changes in consumers' needs. Modern consumers, in fact, express different dietary needs, tastes and preferences compared to the past. In recent decades, consumers have shown greater attention to the environment, aware that human health cannot be separated from environmental health (Lusk et al., 2017). Moreover, there is also a greater focus on nutrition and its effect on consumer wellness (De Canio et al., 2021). As a result, consumers' demand for food that is sustainably produced, healthy, free of chemical additives and with a longer shelf-life is increasing (Li et al., 2021). Often, new ideas are generated by universities, which are the engine of innovations. Outputs of innovation are represented by new processes, products, and markets, that are developed in a scale, the laboratory scale, often far from the real industrial scale. The technology transfer of the innovation, which is defined as the process of transforming research findings into viable outputs that can be commercialized, is the weak point of the process. Gachanja (2023) showed that technology transfer from universities to food firms is fundamental to achieve innovation. As a result, universities and food industries should cooperate, overcoming divergent attitudes between them and also, the SDGs mentioned technology transfer as a significant process to achieve sustainable development (Corsi et al., 2020). In this context, the aim of this doctorate project, partially (50%) funded by the company Matarrese srl, sited in Alberobello (Bari, Apulia, Italy) will be the implementation of a model for the scale-up of food innovative solutions proposed by research, on a scale closer to the industrial one. Food prototypes developed at laboratory level (Technology Readiness Level, TRL 2-3), by the department of the Food science and technology Unit of the University of Bari Aldo Moro in collaboration with international research partners, will be validated and designed at the industrial level (TRL 5-6) in collaboration with Matarrese srl. The design and transfer model will be developed and applied both for meat-based products (low fat burgers with shelf-life extended) and vegetable-based ones (fresh pasta enhanced with free and microencapsulated olive pomace extracts and bakery products obtained by unconventional ingredients, such as legume flours and protein concentrate and acorn flours).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following work packages and activities according to the Gantt diagram given in Table 1:

A0) **Bibliographic research**

WP1: Development and scale-up of meat products with improved nutritional characteristics

A1.1- Design of low-fat burgers with extended shelf-life and characterization

A1.2 - Industrial scale-up of production processes and consumers acceptability tests

WP2: Development and scale-up for innovative vegetable products

A2.1 Scale-up of innovative formulations of gluten-free and gluten content flat bread:

A2.1.1 Scale up of products already developed in laboratory scale

A2.1.2 Assessment of consumer acceptability of gluten free and gluten content flat bread

A2.2: Scale-up of fresh pasta enhanced with vegetable by-products:

A2.2.1: Scale up of fresh pasta (already developed in laboratory scale) enhanced with free and microencapsulated olive pomace extract.

A2.3 - Valorization of acorn flour for food use- this activity will include:

A2.3.1 - Sampling and characterization of acorn flours

A2.3.2 - Evaluation of primary and secondary shelf-life of acorn meal

A2.3.3 - Development of laboratory-scale prototypes of bakery products with acorn flours and characterizzation

A2.3.4 - Scale up of products and assessment of consumer acceptability

A3-Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A0)	**Bibliographic research**	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
WP1	**Development and scale-up of meat products with improved nutritional characteristics**																								
	A1.1- Performance of laboratory tests and qualitative characterization of products		■	■							■	■	■	■	■	■	■	■							
	A1.2-Industrial scale-up of production processes				■	■	■	■	■	■									■	■	■	■	■	■	■
WP2	**Development and scale-up for innovative plant products**																								
	A2.1-Development and scale-up of innovative formulations of gluten-free and gluten content flat bread																								
	Scale up of products already developed in laboratory scale	■	■	■						■	■	■	■	■	■	■	■	■	■						
	Assessment of consumer acceptability of gluten free and gluten content flat bread													■	■	■	■	■	■						
	A2.2 - Scale-up of fresh pasta enhanced with vegetable by-products																								
	Scale up of fresh pasta enhanced with free and microencapsulated olive pomace extract	■	■	■																					
	A2.3 - Valorization of acorn flour for food use																								
	Sampling and characterization of acorn flours		■	■							■	■	■	■	■	■	■	■	■	■	■				
	Evaluation of primary and secondary shelf-life of acorn meal													■	■	■	■	■	■	■	■				
	Development of laboratory-scale prototypes of bakery products with acorn flours and characterizzation													■	■	■	■	■	■	■	■				
	Scale up of products and assessment of consumer acceptability													■	■	■	■	■	■	■	■				
A3	**Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

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Development of a platform to evaluate plant bioactive intake in the population and predictive models for dietary assessment using intake biomarkers.

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This PhD thesis research project aims to evaluate the intake of dietary plant bioactives in the population through the development of a new comprehensive food composition database. It will take into consideration different types of phytochemicals, belonging to major compound families that may have relevant effects on our health, like (poly)phenols, terpenoids, *N*-containing compounds and miscellaneous phytochemicals. Then, predictive models for dietary assessment using intake biomarkers will be created.

Sviluppo di una piattaforma analitica per determinare il consumo di composti bioattivi vegetali nella popolazione e metodi predittivi per la valutazione dietetica utilizzando biomarcatori di consumo alimentare.

Questo progetto di tesi di dottorato ha lo scopo di valutare il consumo di composti bioattivi vegetali provenienti dalla dieta nella popolazione, attraverso lo sviluppo di un nuovo e comprensivo database di composizione alimentare. Verranno presi in considerazione diversi tipi di molecole bioattive, appartenenti alle principali famiglie di composti che possono avere effetti rilevanti sulla nostra salute, come (poli)fenoli, terpenoidi, composti contenenti azoto e fitocomposti misti. Successivamente, verranno creati modelli predittivi per la valutazione dietetica, utilizzando biomarcatori di consumo.

1. State of the art

Healthy dietary habits are one of the most important factors to reduce chronic non-communicable diseases (NCDs) and all-cause mortality (Afshin *et al*., 2019). The consumption of fruit and vegetables is related to a decrease of several pathologies, like cardiovascular diseases and some types of cancer (Willett and Stampfer, 2013). This is mainly due to the presence in these foods of different components like fibres, micronutrients and many bioactive compounds that can help in the prevention of chronic diseases (Liu, 2013). (Poly)phenols and carotenoids are the most studied compounds (Liu, 2013) while there is a lack of information about many others, like phytosterols, glucosinolates, alkaloids, thiosulfinates, and alkylresorcinols. These phytochemicals are showing increasing evidence in the promotion and maintenance of a good health status, even if only results from *in vitro* studies have been published for some of them (Fraga *et al*. 2019; Landberg *et al*., 2014). It is also important to consider that there could be some factors that can drive inter-individual variability and thus health effects after consumption of these compounds, like age, sex, and genetic polymorphisms (Gibney *et al*., 2019, Milenkovic *et al*. 2017).
For some classes of bioactive compounds, missing data on their content in foods makes it difficult to determine the amount consumed everyday through the diet by the population. Also, food composition databases usually report only data regarding macro- and micro-nutrients and the most consumed compounds. This means that a lot of information about a considerable number of compounds we eat every day is missing. Considering this, more efforts are needed to understand the effects of these bioactives on our health and how they may be used as preventing tools against non-communicable diseases. Starting from the assessment of the intake of these plant bioactive compounds in our diet can represent a turning point. New methodologies for the evaluation of dietary intake are thus essential to have a complete view on our diet and consequently on the health status. In this sense, biomarkers of intake may also help to create more objective methods for dietary assessment, limiting bias related to traditional methods (Garcia-Aloy *et al*., 2017).

2. PhD Thesis Objectives and Milestones

Taking into account all issues discussed above, this PhD project can be divided into major points:

1) **Development of a food composition database about dietary plant bioactives.** This part of the PhD project is the most complex and time consuming. It lasted the first year and will be part of the second yeat

too. It consists in the creation of interconnected food composition databases on the most important plant bioactives, like (poly)phenols, terpenoids, *N*-containing compounds and miscellaneous phytochemicals.

2) **Determination of the dietary plant bioactive intake in different populations.** Using the developed database, it will be possible to assess the intake of several phytochemicals in many populations, using data both coming from studies conducted by the University of Parma or their collaborators and publicly available. Finally, comparisons among different cohorts will be done to provide a global picture of the intake of plant bioactives.
3) **Development of predictive models for dietary assessment using data on the intake of plant bioactives and intake biomarkers**. Results coming from the intake of dietary plant bioactives in different populations and data regarding intake biomarkers will allow us to demonstrate the relationship between them and to develop new predictive models for an objective dietary assessment.
4) **Writing and editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1)	***Development of a food composition database about dietary plant bioactives***																								
2)	***Determination of the dietary plant bioactive intake in different populations***																								
	1) Intake assessment in different population settings																								
	2) Comparison between different populations																								
3)	***Development of predictive models for dietary assessment using data on the intake of plant bioactives and intake biomarkers***																								
4)	***Thesis and Paper Preparation***																								

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The role of biomass in the process of ecological transition: characterization and comparison of traditional and renewable energy sources

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This doctoral research intends to highlight modern trends and approaches in replacing fossil fuels for global energy needs. The project aims to examine the potential of renewable energy and the current situation of renewable energy-related industries through an overview of production processes and control strategies in renewable energy production technology, thus contributing to the acquisition of essential information that goes in the direction of improving and optimizing these energy sources. In particular, it will focus on the potential achievement of defined goals and targets for sustainable development to meet environmental, social and economic needs. The environmental impacts generated by renewable energy, compared to traditional fuels, will also be characterized and compared.

Il ruolo delle biomasse nel processo di transizione ecologica: caratterizzazione e confronto tra fonti energetiche tradizionali e rinnovabili

Questo progetto di dottorato mira a mettere in risalto tendenze e approcci moderni nella sostituzione dei combustibili fossili per il fabbisogno energetico globale. Il progetto si propone di esaminare il potenziale delle energie rinnovabili e la situazione attuale delle industrie legate alle energie rinnovabili, attraverso una panoramica dei processi di produzione e delle strategie di controllo nella tecnologia di produzione delle fonti energetiche rinnovabili, contribuendo così all'acquisizione di informazioni essenziali che vanno in direzione del miglioramento e dell'ottimizzazione di queste fonti energetiche. In particolare, si concentrerà sul potenziale raggiungimento di obiettivi e traguardi definiti per lo sviluppo sostenibile per soddisfare le esigenze ambientali, sociali ed economiche. Verranno inoltre caratterizzati e confrontati gli impatti ambientali generati dall'energia rinnovabile, rispetto ai combustibili tradizionali.

1. State-of-the-Art

In recent years, human population growth and overall industrial development have led to an exponential increase in global energy demand (Kang et al., 2020). The European economy still heavily relies on fossil fuels for its energy needs, but these will be depleted in the coming decades. An economy based on renewable and low-cost energy sources, a clean environment, and energy independence are characteristics of responsible societies, and all of these have become the global challenge of our time. Commitments related to climate policy and the energy crisis have led to a search for alternative ways to obtain energy. One element of this vision is to replace traditional fossil fuels with biofuels. European Union energy policy reform and subsequent legislation and regulation at the national level have provided a strong incentive for the development of increasingly competitive renewable energy generation technologies. Europe, with its European Green Deal strategy, aims to become the first climate-neutral continent by 2050 (Cambini et al., 2020).
Bioenergy is considered the most consistent renewable energy source because of its economic advantages and its great potential to replace non-renewable fuel sources. In the current scenario, renewable technologies produce electrical, thermal or mechanical energy using biomass (energy crops, agricultural or forest residues, urban waste, etc.), wind, solar (thermal and photovoltaic), hydroelectric (river flow, tide, wave motion) and geothermal energy are considered the best alternative sources (Kasinath et al., 2021). The production of energy from biomass has shown significant growth and an increasing weight among renewable energies (Magazzino et al., 2022); biomass is the most abundant renewable resource on earth (Schen et al., 2020) and it is considered the leading emerging alternative to fossil fuel resources. Moreover, it can provide energy and multiple products (Awasthi et al., 2020). In fact, it will play an increasingly important role in the future global energy infrastructure, for the generation of electricity and heat, but also for the production of gaseous and liquid fuel products (Kasinath et al. 2021; Speight et al., 2020). Efficient conversion of biomass into energy requires investment in research and development of innovative technologies. The knowledge of the biological and technical mechanisms is essential to favor the introduction of more advanced and efficient techniques/methods in terms of energy, economic and environmental sustainability. Many are the processes that can be used to convert biomass into energy: it can be burned, transformed into fuel gas by partial combustion, biogas by fermentation, alcohol by biochemical processes, biodiesel, bio-oil, or syngas from which chemicals and fuels can be synthesized.
Circular economy and bioeconomy are the approaches to address the challenges and achieve global environmental and socio-economic goals (Stark et al., 2022; Biber-Freudenberger et al., 2018). Therefore, the generation of energy from alternative sources will contribute to the mitigation of climate change and to minimizing the alarms posed to the environment.

2. Ph.D. Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Bibliographic research and evaluation of the state of the art**.

A2) **Study of use of biomass as a renewable resource:** to identify the common biomass sources (A2.1) and methods of converting biomass into energy (A2.2). The opportunities and challenges of the obtained products will also be established (A2.3).

A3) **Evolution and current regulation of global European policies:** decarbonisation policies (A3.1) and adoption of sustainable and reliable energy systems(A3.2).

A4) **Characterization and comparison of the environmental impacts of the use of biomass with respect to traditional energy sources**, through the inventory of the use of traditional and renewable energies (A4.1) and the energy and environmental comparison between traditional and alternative sources (A4.2).

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity ——— Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Bibliographic research and evaluation of the state of the art***																								
A2)	***Study of use of biomass as a renewable resource***																								
	1) Common biomass sources																								
	2) Methods of converting biomass into energy																								
	3) Opportunities and challenges of the products obtained																								
A3)	***Evolution and current regulation of global European policies***																								
	1) Decarbonisation policies																								
	2) Adoption of sustainable and reliable energy systems																								
A4)	***Characterization and comparison of the environmental impacts of the use of biomass with respect to traditional energy sources***																								
	1) Inventory of the use of traditional and renewable energies																								
	2) Energy and environmental comparison between traditional and alternative sources																								
A5)	***Thesis and Paper Preparation***																								

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Lactic acid bacteria fermentation to improve the techno-functional and nutritional value of products and by-products in the agri-food chain

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The purpose of this PhD research project is to investigate the potential use of lactic acid bacteria (LAB) fermentation to valorise the tecno-functional characteristics of product and by product of food industries. The first part of the research will be focused on the study of different LAB metabolism with the aim to find out the most performing strains to be used in the fermentation process. In the last part of the project the fermentation will be used as part of the production process to obtain a product that will be reinsert in the food chain.

Fermentazione lattica come strategia per migliorare le caratteristiche tecniche, funzionali e nutrizionali di prodotti e sottoprodotti della filiera agroalimentare

L'obbiettivo di questo progetto di dottorato è di investigare il potenziale uso della fermentazione da parte di batteri lattici (LAB) per valorizzare le caratteristiche tecnologiche, funzionali e nutrizionali dei prodotti e sottoprodotti della filiera agroindustriale, con l'obbiettivo finale di ottenere prodotti tradizionali migliorati e/o prodotti innovativi. La prima parte della ricerca sarà focalizzata sullo studio del metabolismo dei LAB, al fine di trovare i ceppi più performanti. Successivamente, la fermentazione sarà usata come parte integrante del processo per ottenere prodotti che saranno reinseriti nella filiera agroalimentare.

1. State-of-the-Art

There is a lot of evidence showing how the overuse of our natural resources is taking us to a point of no return where the environment can no longer sustain society as we know (Aschemann-Witzel and Stangherlin, 2021).
By 2050, there will be at least 10 billion people in the world, and the demand of food sources is projected to significantly increase and, alongside this, food waste (FW) is becoming one of the most severe environmental, social, and economic problems in developed and developing countries. That's why moving to more sustainable protein sources together with the optimization of the current resources and the reduction and re-use of food waste throughout the agri-food chain, has become of primary importance.
However, using this kind of matrices is a real challenge, because their chemical composition, the presence of antinutritional compounds, the unavailability of important components such as vitamins and often the poor techno-functional and organoleptic properties, make these kinds of products difficult to use.
Research is fast moving forward to find innovative and sustainable technologies to reuse and integrate new protein sources and food waste in the food chain.
In this optic, lactic acid fermentation could represent a valid tool to improve the techno-functional properties, making these matrices more attractive (Adebo et al., 2022; Papagianni, 2012).
It is known that LAB are able to produce high-valuable molecules and, thanks to their metabolism, could modify the structural characteristics of the matrices through proteolysis and the production of viscous compounds such as exopolysaccharides (EPS) (Wang et al., 2021). In particular, in this PhD project, the phenotypic characteristic of different LAB species belonging to UPCC (University of Parma Culture Collection) will be studied and the most promising strains will be selected and used for the fermentation process.
In particular, the fermentation characteristics, the ability to produce exopolysaccharides and thus the possibility to modulate the viscosity of fermented products, together with the ability to produce aroma and other interesting compounds will be taken into consideration.
Different vegetal-based products will be tested, from plant food waste, plant by-products, plant protein extract, cereals and legumes flours, etc.
The final aim of this PhD project will be to improve the characteristics of existing products by adding the fermented matrices as a functional ingredient or developing new fermented food and/or beverages from FW and underutilize matrices, that will be reinsert in the food chain.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Literature review**
A2) **Screening of LAB,** grow performances and metabolites production
A3) **LAB fermentations,** use of underuse traditional or alternative sources from different vegetable matrix
A4) **Developing new enriched products** or renovate traditional foods

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	**Literature review**																								
A2)	**LAB screening**																								
A3)	**LAB fermentations**																								
A4)	**Developing new enriched products**																								
A5)	**Thesis and Paper Preparation**																								

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Sourdough Fermentation As a Tool to Increase the Nutritional properties of Leavened Baked Goods

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This PhD thesis research project aims to develop a functional sourdough bread with low gluten, improved nutritional, and biochemical properties using a consortium of probiotic strains. The *in vitro* study of digestion of the low gluten sourdough bread will also be conducted on validated digestion model. This study is expected to shed light on the potential nutritional benefits of sourdough bread made with specific probiotic strains and may have implications for dietary recommendations for gluten sensitive populations.

La fermentazione del lievito madre come strumento per aumentare le proprietá nutrizionali dei prodotti lievitati da forno

Il progetto di ricerca di tesi di dottorato ha lo scopo di sviluppare un pane funzionale a lievitazione naturale con basso contenuto di glutine, migliori proprietà nutrizionali e biochimiche utilizzando un consorzio di ceppi probiotici. Lo studio *in vitro* della digestione del pane a lievitazione naturale a basso contenuto di glutine sarà condotto anche su un modello di digestione validato. Questo studio dovrebbe far luce sui potenziali benefici nutrizionali del pane a lievitazione naturale prodotto con specifici ceppi probiotici e, potrebbe avere implicazioni per le raccomandazioni dietetiche per le popolazioni O sensibili al glutine

1. State-of-the-Art

Cereal fermentation is one of the oldest biotechnological processes, dating back to ancient Egypt, where both beer and bread were produced by the help of yeasts and lactic acid bacteria (LAB). Initially, spontaneous fermentation was used just to activate the naturally occurring microbes in milled grains. In the more recent past, the use of sourdough has already been more systematic, sustainable, and effective tool for ensuring hygiene, rheology, sensory and shelf-life features, and improving the functional/nutritional value of many animal- and plant-based foods and beverages (Gobbetti et al., 2019).

The focus of sourdough research was mostly directed on the technological effects of sourdough on baked goods such as how it affects taste, texture, and shelf life, as well as the microbial interactions involved in the process. However, in recent years scientific research has also moved towards the functional and /nutritional features of sourdough fermentation. Sourdough fermentation boasts a plethora of health benefits. It has been reported to lower the glycemic index, enhance its fiber availability, and release bioactive compounds that are beneficial for human health. Furthermore, released organic acids can aid digestion, reduce inflammation, and improve mineral absorption (Gobbetti et al., 2019). Furthermore, the microbial metabolism of *Lactobacillus* spp. harboring the sourdough produces nutritionally bioactive compounds, such as amino acid derivatives and potentially prebiotic substances. Scientists are keen to explore the potential of developing new products that can assist in managing chronic ailments such as high cholesterol, heart disease, autoimmune disorders, and diabetes (Canesin and Cazarin, 2021).

Sourdough *Lactobacillus spp.* enhance the activity of cereal proteases, allowing gluten to break down more easily. As a result, amino acids accumulate, which can be further broken down by specific intracellular peptidases found in lactobacilli. Proteases in the fermentation process can extensively degrade proteins in sourdough, creating new products that are safe for individuals with gluten intolerance. Thus, proteolysis performed by LAB proteases has been suggested as a new tool for food processing for celiac persons (Siepmann et al., 2018).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Optimization of sourdough process**
To optimize the sourdough process, we will employ various propagation techniques at different fermentation times. This will involve conducting microbial and biochemical analyses to effectively achieve our objective.

A2) **Enzymatic treatment of sourdough**
The sourdough will also be treated with commercial protease enzyme to identify its effect on protein, texture, and

sensory properties.

A3) **Characterization of the optimized functional sourdough and bread**
Once the sourdough process has been optimized, we will proceed to prepare sourdough bread. Both the sourdough itself and the resulting sourdough bread will undergo characterization to assess their biochemical, and nutritional properties, respectively.

A4) ***In vitro* digestion study**
To investigate the effects of low gluten sourdough bread on the digestive tract (including the oral cavity, stomach, and small intestine), a validated in vitro digestion model will be employed. This model will be utilized to assess the optimal operating conditions for the digestion process. The evaluation will focus on examining the impact of low gluten sourdough bread on the digestive tract, with the aim of identifying potential health benefits specifically for individuals with gluten intolerance or sensitivity.

A5) **Writing and Editing**
Writing and editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Optimization of sourdough process***																								
	1) Preparation and propagation at different fermentation time																								
	2) LAB persistence experiment																								
A2)	***Enzymatic treatment***																								
	1) Microbial characterization																								
	2) biochemical characterization																								
A3)	***Characterization of the optimized functional sourdough and bread***																								
	1) Biochemical Characterization																								
	2) Nutritional Characterization																								
A4)	***Invitro digestion***																								
A5)	***Thesis and Paper Preparation***																								

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DEVELOPMENT OF NEW MICROBIAL BIOSTIMULANTS FOR THE SUSTAINABLE PRODUCTION OF FOOD CROPS

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This PhD thesis research project is aimed to obtain new commercial formulations starting from newly isolated microorganisms, with plant growth-promoting activity capable of stimulating plant growth in water or saline stress conditions. The formulations obtained, developed with a view to eco-sustainable agriculture, could represent an innovative solution to increase the yield of plants of food interest and reduce the negative impact that climate change and drought have on our production.

Sviluppo di nuovi biostimolanti microbici per la produzione sostenibile di colture a interesse alimentare

Questo progetto di tesi di dottorato mira ad ottenere nuove formulazioni commerciali a partire da microrganismi appena isolati, con attività fitopromotrice in grado di stimolare la crescita delle piante in condizioni di stress idrico o salino. I formulati ottenuti, sviluppati nell'ottica di un'agricoltura ecosostenibile, potrebbero rappresentare una soluzione innovativa per aumentare la resa delle piante di interesse alimentare e ridurre l'impatto negativo che il cambiamento climatico e la siccità stanno avendo sulle nostre produzioni.

1. State-of-the-Art

The Green Revolution, initiated in the 1970s, played a pivotal role in increasing agricultural production through mechanization and chemicals. The utilization of synthetic fertilizers and pesticides boosted crop productivity and averted global famine. However, modern agriculture heavily reliant on the indiscriminate use of synthetic chemicals has had adverse effects on soil, the environment, and human health. Furthermore, abiotic stresses such as drought, heat, and pollution impact plant growth, development, and productivity (Naik et al. 2019). In recent years, there has been a pursuit of eco-friendly agricultural practices to promote plant growth and productivity. Among emerging strategies, biostimulants are emerging as a cornerstone for a new agricultural revolution towards sustainable food production (Nephali et al. 2020). Biostimulants are products that stimulate plant nutritional processes to enhance various plant or rhizosphere characteristics, such as nutrient use efficiency, abiotic stress tolerance, quality traits and nutrient availability in soil or rhizosphere. The recent literature demonstrates that the ability to promote plant growth is associated with two main groups: microbial biostimulants, with several bacteria such as Plant Growth-Promoting Rhizobacteria (PGPR) and fungi and non-microbial biostimulants, such as algae extracts, humic substances, protein hydrolysates, and other biopolymers. PGPR are microorganisms that promote plant growth and yield through direct and indirect mechanisms. Indirect mechanism affects plant health by protecting against pathogens through the production of siderophores, antibiotics, cell wall degrading enzymes, and soluble and volatile metabolites. Direct plant growth-promoting mechanisms positively affect the availability of essential nutrients (e.g., nitrogen, phosphorus, iron) and produce and regulate compounds involved in plant growth (e.g., phytohormones) (Luziatelli et al. 2023). Among plant regulators, indole-3-acetic acid (IAA) is the most abundant member of the auxin family. The effects of biostimulants are partially regulated by hormonal changes in plants (Ruzzi et al. 2015). Phytohormones, such as auxins, are involved in growth promotion by PGPR. Biostimulants can also emit volatile organic compounds that support plant development and induce pathogen resistance.

The utilization of PGPR microorganisms is a promising strategy to improve agricultural productivity. These microorganisms can be used as biocontrol agents, biofertilizers, and biostimulants, and they can also contribute to the bioremediation of contaminated environments. The combination of different microorganisms in a formulated product can promote synergistic interactions and enhance plant growth.

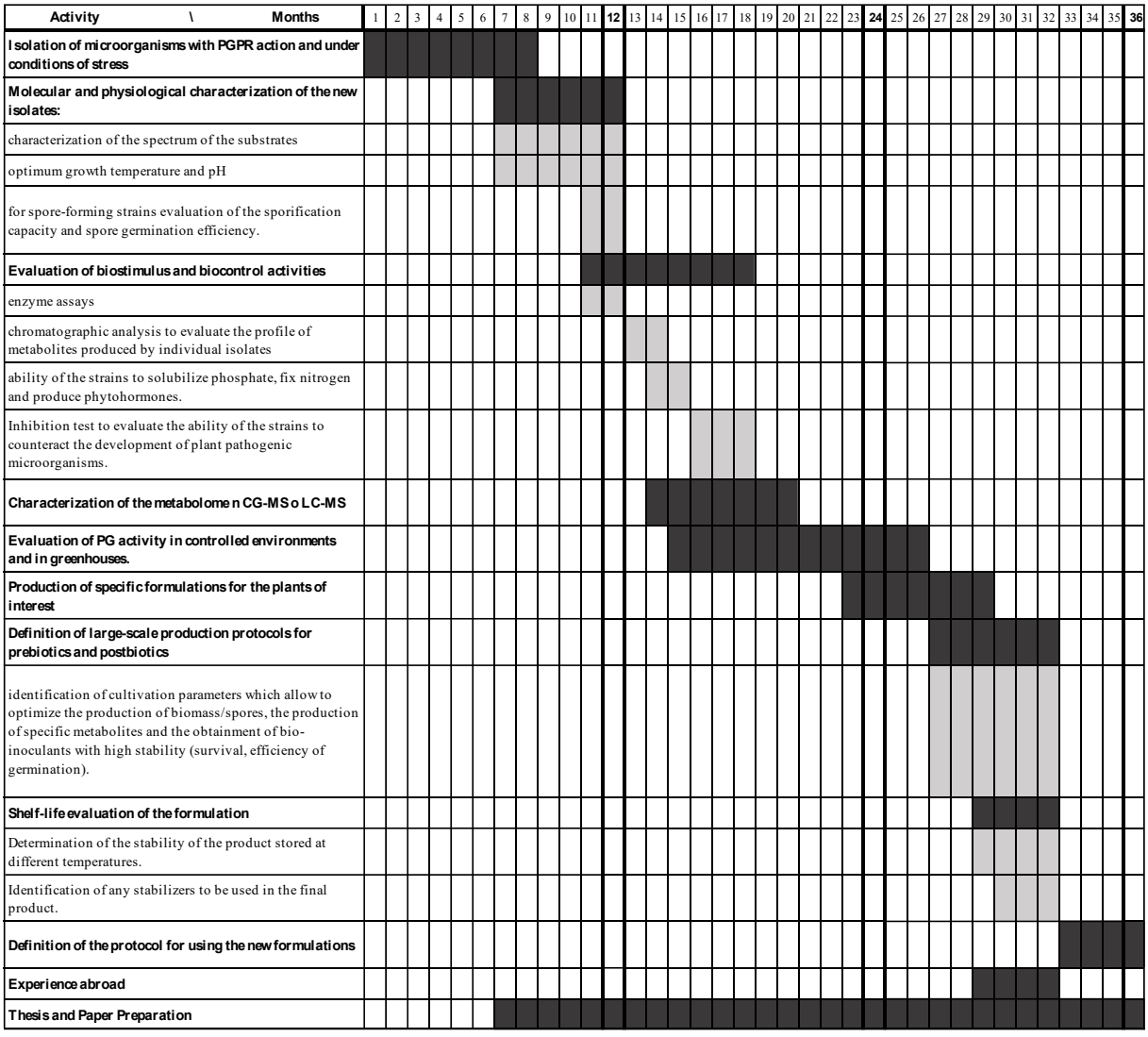
2. PhD Thesis Objectives and Milestones

In detail, the objectives of this research activity aim to:
• Isolate microorganisms (bacteria and fungi) from different natural habitats with plant-promoting activity.

• Taxonomically characterize the strains with innovative phenotypic and sequencing approaches.
• Characterize the metabolome of the isolated strains with the use of chromatographic techniques to fully understand the spectrum of action and obtain formulations with different combinations of secreted metabolites.
• Develop an optimized protocol for the industrial production of different prebiotics and postbiotics.
• Drafting and planning a brochure that highlights the methods of use and conservation of the formulation

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1.

Table 1: *Gantt diagram for this PhD thesis project.*



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Assessment of Organic Amendments on Vegetative and Reproductive Performance of Strawberry Plants Under Different Growing Conditions

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The Ph.D. project aims at investigating the role of natural amendments and biostimulants of different origins and at different concentrations, on the vegeto-productive response of strawberry plants, grown in vivo (in pots and in the field) and in vitro. Mainly, morphological, physiological, biochemical, and nutritional quality attributes of plants and fruits will be evaluated to individuate the best combination of strawberry genotype, substrate composition, and growing conditions.

1. State-of-the-Art

The growth of the global population and the improvement in living conditions have led to an increase in the demand for food supply, to fulfil which agriculture has impacted strongly on the environment, and, moreover, has represented a potential health hazard to people; in fact, there has been an increasing recourse to synthetic products, such fertilizers and pesticides, but, at same time, recent years have seen an increment of agri-food waste, with about 2 billion tons generated annually worldwide (Wu *et al.,* 2020; Khan, 2022; Chhandama, 2022). To increase the agricultural sustainability, a strategy could be the reutilization of agri-food waste in agriculture to increase soil fertility, to reduce the use of chemical fertilizers and to improve plant vegeto-productive performances (Akram *et al.,* 2023); recently, there has been a fruitful scientific production about the use of natural and eco-friendly organic amendments, like biochar, and biostimulants (Mousavi-Avval *et al.,* 2023). Biochar derives from the anaerobic pyrolysis of agricultural waste, and it is known to improve the overall soil quality, microbial and enzymatic activity, and soil organic carbon content with nutrient retention and water availability (Elkhlifi *et al.,* 2023). Biostimulants are substances or microorganisms that are used to improve the nutrient uptake, to promote plant growth (germination, flowering, pollination, fructification, maturity, and crop quality), and to protect plants from biotic and abiotic stresses (Hijri, 2023); they trigger plant growth by solubilizing minerals, nitrogen fixation, and introducing phytohormones, secondary metabolites, volatile organic compounds, and lipopolysaccharides (Caulier *et al.,* 2019). Strawberry (*Fragaria×ananassa* L.) is highly appreciated for its high organoleptic and nutraceutical qualities, since it is rich in phenolic compounds, vitamins, and minerals (Garza-Alonso *et al.,* 2022). Biochar, alone or in combination with biostimulants, has already been applied to strawberry cultivation, enhancing, in the fruits, the enzymatic activity, the total soluble solid and the phenolic compound content, the total flavonoids, the beta-glucosidase activity and the phosphatase activity also (Shang *et al.,* 2021; Chiomento *et al.,* 2022). Other than in traditional agricultural systems, the use of biochar and biostimulants could be of enormous impact in strawberry tissue culture; in fact, micropropagation is highly dependent upon the use of plant growth regulators which may often be expensive and may cause aberrant and undesirable physiological and epigenetic disorders; moreover, toxic chemicals could be produced. Adding biostimulants to culture medium would help in replacing, *in toto* or partially, phytohormones, given their hormone-like properties; moreover, biochar can be a valid substitute of activated charcoal, for toxic compound absorption (Masondo *et al.,* 2022).

2. PhD Thesis Objectives and Milestones

The objectives and the different activities of the study are the following:

A1) **Evaluation of biochar and biostimulants in *in vitro* strawberry tissue culture** vegeto-productive and physio-chemical response of in vitro plants will be monitored.

A2) **Evaluation of different types and concentrations of biochar and wood distillate in strawberry cultivation in pots** morpho-physiological, physico-chemical response of strawberry plants grown in pots with different types and concentrations of biochar and wood distillate will be evaluated.

A3) **Evaluation of different types and concentrations of biochar and wood distillate of strawberry cultivation in field** morpho-physiological, physico-chemical response of strawberry plants grown in field with different types and concentrations of biochar and wood distillate will be evaluated.

A4) **Writing and Editing** the PhD thesis, scientific paper and oral or poster communications.

Timetable of proposed activities are summarized in the Gannt charts in Table 1.

Table 1. Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Evaluation of Biochar and Biostimulants in In Vitro Strawberry Tissue Culture***																								
A2)	***Evaluation of Different Types and Concentrations of Biochar and Wood Distillate in Strawberry Cultivation in Pots***																								
A3)	***Evaluation of Different Types and Concentrations of Biochar and Wood Distillate of Strawberry Cultivation in Field***																								
A4)	***Writing and Editing of Ph.D. Scientific Paper and Oral or Poster Communication***																								

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Development of an active bio-based coating for corrugated cardboard with waterproofing and antimicrobial properties

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Tutor: Prof. Rosalba Lanciotti
Co-tutors: Dr. Lorenzo Siroli and Dr. Claudio Dall'Agata

The present research project aims at developing, during the three-year period of the industrial PhD, a corrugated cardboard packaging coated with a bio-based film which is obtained from bacterial cellulose produced by selected strains of acetic acid bacteria and functionalized thanks to the addition of natural antimicrobial agents, capable of reducing microbial proliferation and increasing the shelf-life of fruits and vegetables.

Messa a punto di un coating attivo bio-based per cartone ondulato ad azione impermeabilizzante e antimicrobica

Il presente progetto di tesi si propone di mettere a punto, nel triennio del dottorato industriale, un imballaggio in cartone ondulato rivestito da un film bio-based, ottenuto a partire da cellulosa batterica prodotta da ceppi di batteri acetici selezionati, e attivato grazie all'addizione di antimicrobici naturali rilasciati nel tempo, in grado di ridurre la proliferazione microbica e di incrementare la shelf-life di prodotti ortofrutticoli confezionati.

1. State-of-the-Art

The reduction of food wastes and the negative environmental impact of fossil-based plastic used in the food packaging industry are certainly among the most debated topics nowadays. The emphasis was placed on these key issues by including them in the 2030 Agenda for Sustainable Development of the United Nations. In fact, more than 40% of the global production of plastic materials are used for packaging and approximately 96% of this material is converted into waste and is not recycled (Alshehrei, 2017; Ncube *et al*., 2021). There is then, a huge and urgent need for more sustainable materials with added properties (such as antimicrobial activities, improved mechanical and thermal properties).
In order to reduce the dependence of the food industry on non-renewable resources such as Petroleum based polymers, research efforts have been developed at different levels to find alternative solutions which include the production of more sustainable packaging using biodegradable and renewable polymers (Cazón and Vázquez, 2021; Wang *et al*., 2022). For instance, biopolymers such as alginate, chitosan, lipid-based compounds or those based on proteins (egg white, casein, soy protein, collagen, gluten, whey protein, fish gelatine, myofibrillar proteins) have been successfully tested and the results highlighted the great potential of these biomaterials to replace synthetic polymers in the food packaging industry (Wang *et al*., 2022). However, among the bio-based materials, polysaccharide-based polymers have received tremendous attention due to their low production cost, biodegradability, wide availability and broad application (Xu *et al*., 2016). On the other hand, numerous studies have highlighted the possibility of using compounds deriving from microbial biomass or microbial metabolism such as bacterial cellulose, produced by some strains of acetic acid bacteria such as *Komagataeibacter* spp. and *Novacetimonas* spp., to produce food packaging and coatings with technological properties almost similar to those of traditional packaging and with the great additional advantage of being biodegradable (Kolesovs *et al*., 2022; Cazón and Vázquez, 2021). Furthermore, bio-based packaging intended to come into contact with food can, in some cases, be functionalized through the incorporation of natural components with antimicrobial or antioxidant activity which can be gradually released into the food product or its environment, allowing an increase in the shelf-life of the product and consequently the reduction of food wastes and losses (Almasi *et al.*, 2021). Despite the large number of studies completed on this topic, the development of sustainable and effective functional biobased coating for food packaging applications is still far to be reached. For this reason, the present industrial PhD research project, in partnership with Bestack (Italian consortium of corrugated cardboard packaging), is aimed to develop an active bio-based coating for cardboard packaging, obtained from bacterial cellulose produced by selected acetic acid bacteria strains, and activated by the addition of natural antimicrobials released over time, able to reduce the microbial proliferation and increase the shelf-life of packaged fruits and vegetables.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Bibliography research**

A2) **Selection and production of bio-based films using acetic acid bacteria**: this first step aims to isolate and identify acetic acid bacteria from different food matrices, especially wastes or by-products (A2.1) and then, screen and characterize them for bacterial cellulose production (A2.2). The cellulose production by the selected strains will be improved and optimized by the modulation of culture parameters (A2.3) and the optimal protocol for bacterial cellulose production will be defined (A2.4).

A3) **Selection of natural antimicrobials for the activation of bio-based films**: the second phase aims at screening and characterizing natural antimicrobial agents obtained from different sources such as essential oils and antimicrobial peptides (A3.1). In particular, the considered compounds will be characterized for organoleptic compatibility and for their antimicrobial activity against foodborne pathogens and spoiling microorganisms associated with fresh fruits and vegetables. Those of greatest interest will be selected (A3.2) and used for packaging activation.

A4) **Activation and characterization of bio-based films**: definition of protocols and ingredients for the production of biopolymers integrated with natural antimicrobials (A4.1), characterization of the antimicrobial activity of the active bio-based film (A4.2) and technological characterization of the bio-based film obtained.

A5) **Application on corrugated cardboard and evaluation of the effect on a packaged product**: definition of the optimal protocol for the coating of corrugated cardboard with the obtained functional bio-based film (A5.1), evaluation of the effect of the active packaging on the shelf-life, safety and organoleptic properties of packaged fruits and vegetables (A5.2) taking into consideration the final requirements of the industrial partner.

A6) **Preparation** of the final PhD thesis, scientific articles, posters and/or oral presentation.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1)	***Bibliography research***																		
A2)	***Bio-based films by acetic acid bacteria***																		
	1) Acetic acid bacteria isolation																		
	2) Screening and characterization																		
	3) Performance optimization																		
	4) Optimal protocol definition																		
A3)	***Natural antimicrobial agents selection***																		
	1) Screening and characterization																		
	2) Antimicrobial activity evaluation																		
A4)	***Bio-based film activation and characterization***																		
	1) Optimal protocol definition																		
	2) Antimicrobial effect evaluation																		
	3) Technological effect evaluation																		
A5)	***Corrugated cardboard coating and evaluation***																		
	1) Optimal protocol definition																		
	2) Effect evaluation on real products																		
A6)	***Preparation of the final thesis, scientific articles, posters and oral presentation***																		

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Assessment of Lipid Oxidation in Food and its Effects in Living Organisms

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This PhD project aims to assess the degree of lipid oxidation products present in long shelf-life commercial food and to verify the effect of a lifelong consumption of food containing rancid oil in a living organism. This will be tested using *in vivo* models of both *Drosophila melanogaster* (DM) wild type and DM with Parkinson's disease. The effects of this diet will be evaluated on the degree of oxidation of their cells, and, on their microbiota determining if and how this diet may affect the evolution of Parkinson's disease.

Valutazione dell'ossidazione Lipidica degli Alimenti e dei suoi Effetti negli Organismi Viventi

Questo progetto di dottorato mira a valutare il grado di ossidazione dei lipidi presenti negli alimenti commerciali a lunga conservazione e a verificare l'effetto di un consumo sistemico di alimenti contenenti olio rancido in un organismo vivente. Questo sarà analizzato utilizzando il modello *in vivo* di *Drosophila melanogaster* (DM) sia wild type che DM ingegnerizzata per sviluppare la malattia di Parkinson. Verranno valutati gli effetti di tale dieta sul grado di ossidazione cellulare, l'eventuale effetto sul microbiota, analizzando se, e come, questa dieta influenzi l'evoluzione del morbo di Parkinson.

1. State-of-the-Art

Lipid oxidation is a series of reactions that negatively influences the shelf-life of food (Barden and Decker, 2016). There are various factors that could affect lipid oxidation in food, like for example the presence of free fatty acids, transition metals, heme proteins, the degree of fatty acid unsaturation, atmospheric or singlet oxygen, lipoxygenase, and environmental factors such as temperature, light and water activity (McClements and Decker, 2000), but also food technologies used to prepare it (Liu et al., 2023). Lipid oxidation and the products that derive from this process are widely known, what is unknown is the toxicity of this molecules and if or how they affect human's health. Gut microbiota is affected by diet, and by the concentration and the composition of dietary lipids; but the mechanisms by which lipids affect gut microbiota are not well defined (Schoeler and Caesar, 2019). On the other hand, the gut-brain-axis is firmly established (Mayer et al., 2021). For this reason, it could be interesting to study the effect of lipid oxidation products both on the microbiota balance and on the neurodegenerative diseases, that are strongly connected with intestine health (Quigley, 2017). *Drosophila melanogaster* is one of the most widespread *in vivo* models used to study molecular mechanisms, genetic inheritance, and some diseases such as Alzheimer or Parkinson (Nitta and Sugie, 2022). However, these flies have also been used as a model for the study of lipid metabolism because they have many similarities with mammalian metabolism, and it is known that in these animals the lipid metabolism is also involved in neuronal diseases (Huntington's disease, Alzheimer's disease, and Parkinson's disease) (Aditi et al. 2016). Humans consume lipid oxidation products every day, so the aim of this study is to understand if and how rancid oils contained in food induce lipid peroxidation in living organisms (*Drosophila melanogaster*) and if these products modulate evolution of neurodegenerative disease like Parkinson, acting on the microbiota.

2. PhD Thesis Objectives and Milestones

This Project is under joined supervision of University of Camerino and University of Massachusetts and it develops as described in Figure 1.

Phase I, University of Camerino, aiming at:

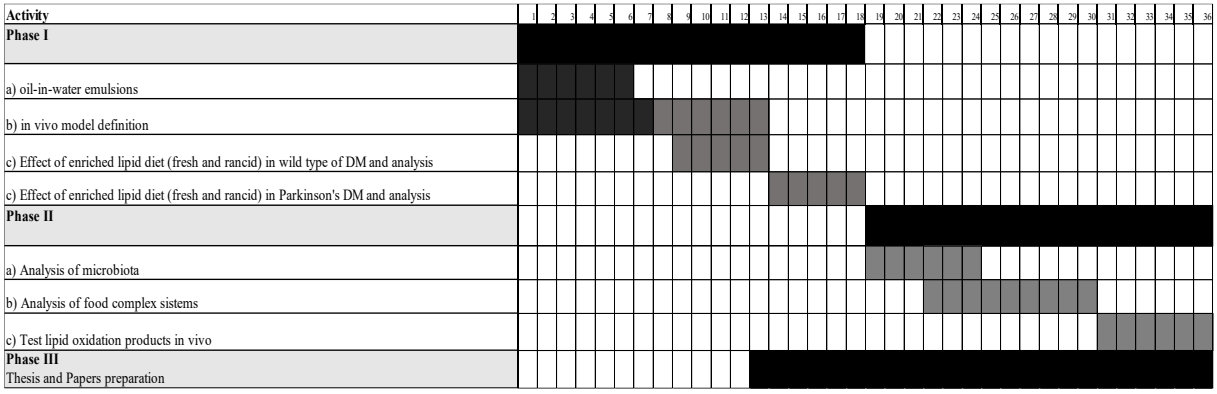
a) Development of protocol for stable emulsions oil-in-water (Extra Virgin Olive Oil fresh and rancid);
b) Verify if it is possible to feed *Drosophila melanogaster* with a lipid enriched diet;
c) Verify the effect of a lifelong lipid (fresh and rancid) enriched diet in wild-type DM on longevity, oxidative stress and expression of genes related to inflammation; collection of gut samples for subsequent analysis of the intestinal microbiota;
d) Verify the effect of a lifelong lipid (fresh and rancid) enriched diet in Parkinson model of DM on longevity, oxidative stress and expression of genes related to inflammation; collection of gut samples for subsequent analysis of intestinal microbiota.

Phase II, University of Massachusetts, aiming at:

a) Analysis of all Drosophila's gut samples to evaluate the abundances of the microbiota;
b) Analysis of food complex systems during storage, evaluation of lipid oxidation products and their toxicity;
c) Testing these products in an *in vivo* model.

Phase III, Writing and editing thesis and papers.

Table 1 Gantt diagram for this PhD thesis project divided in months (from December 2022 to December 2025).



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Multidisciplinary Approach for Authentication and Traceability of Geographical Origin of Agri-food Products

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This PhD thesis research project is aimed at the development, testing and validation of an integrated approach for the traceability of geographical origin and the authentication of quality of agri-food products, which can be adopted in the agri-food chains, useful for the competitiveness of companies.

Approccio multidisciplinare per l'autenticazione e la tracciabilità della provenienza geografica di prodotti agroalimentari

Questo progetto di tesi di dottorato ha come obiettivo lo sviluppo, la sperimentazione e validazione di un approccio integrato per l'autenticazione e la tracciabilità della provenienza geografica di prodotti agroalimentari di qualità, adottabile nelle filiere agroalimentari, utile per la competitività delle aziende.

1. State-of-the-Art

Over the last few years, the identification and certification of the geographical origin of food has gained an increasing amount of attention. Globally, large amounts of food are exchanged and transported on a daily basis, and for many consumers, geographical authentication of food is looked upon as an assurance of its quality and safety (Opatić *et al*., 2017). Therefore, the participation to protected food names (PDO, PGI, TSG) is encouraged in the EU (Luykx *et al*., 2007). The authentication and traceability of the geographical origin of agri-food products is essential to increase consumer confidence in the products and prevent fraud and counterfeiting. The main analytical methods used to implement traceability systems, have been subdivided into four groups: mass spectrometry, spectroscopic, separation, and other techniques (Luykx *et al*., 2007) (Table 1).

Table 1 Main analytical techniques for determination of the geographical origin of food products.

Principle	Main technique
Spectroscopy	Infrared spectroscopy (IR) Fluorescence spectroscopy Atomic spectroscopy Nuclear magnetic resonance spectroscopy (NMR)
Mass Spectrometry	Isotope ratio mass spectrometry (IRMS) Inductively coupled plasma mass spectrometry (ICP-MS) Proton transfer reaction mass spectrometry (GC-MS)
Separation	High performance liquid chromatography (HPLC) Gas chromatography (GC) Capillary electrophoresis (CE)
Others	Sensor technology DNA technology Sensory analysis

These methods can analyse different characteristics of a product allowing to find markers useful for the discrimination of variety, geographical origin, and mode of production. Food traceability usually requires a multi-technique approach as single methods do not generally produce sufficiently discriminating factors (Guyon *et al*., 2020). The set of data obtained provide a characteristic fingerprinting relating to the provenance of the sample. The application of chemometrics is often required to handle the amount of data and to detect subtle differences that frequently exist between food samples (Luykx *et al*., 2007). A multi-analytical approach seems to be the most promising approach for the traceability of geographical origin and the authentication of quality agri-food products.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

1) **Literature review and data collection**.
2) **Food and soil sampling**.
3) **Determination of quality properties**.
4) **Near infrared spectroscopy** analysis as a quick tool to authenticate the origin and quality of agro-products.
5) **Determination of the multi-element fingerprinting** and **stable isotope ratios** (13C/12C, 15N/14N) of agro-products and cultivation soils.
6) **Analysis and study of nutritional composition (macro and micronutrients)** of fresh and processed foods.
7) **Optimization of different chemometric tools** to obtain the development of authentication and traceability models to verify and validate the quality and origins of the samples.
8) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity / Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Literature review and data collection																								
Food and soil sampling																								
Determination of quality properties																								
Near Infrared Spectroscopy																								
Multi-element fingerprinting																								
Stable isotope profiling																								
Study of nutritional composition																								
Chemometrics																								
Thesis and Paper Preparation																								

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Study of the Effects of Plant-Derived MicroRNAs on the Human Gut Microbiota

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This PhD project aims to investigate the stability of plant-derived microRNA (miRNA) throughout the human digestion process, as well as the potential impacts of miRNA on the gut microbiota and their subsequent metabolic responses. In vitro and in-vivo models will be used to evaluate miRNA stability and their impact on the gut microbiota and metabolites. The study has implications for the food and healthcare industries and could contribute to the development of therapeutic treatments.

Studio degli effetti dei microRNA di origine vegetale sul microbiota intestinale umano

Questo progetto di dottorato si propone di valutare la stabilità dei microRNA di origine vegetale assunti dall'uomo con la dieta durante il processo di digestione, così come i suoi potenziali effetti sul microbiota intestinale e la sua risposta metabolica. A tale riguardo, saranno utilizzati modelli in vitro e in vivo. I risultati ottenuti potranno suppportare le industrie alimentari e farmaceutiche nel potenziale sviluppo di nuovi alimenti funzionali e trattamenti terapeutici.

1. State-of-the-Art

The gut microbiota, commonly called our "forgotten organ," with a thriving microbial population of approximately 100 trillion bacteria plays a crucial role in human health and disease. The stability of the intestinal microbiota is directly linked to the well-being and disease of mammals. Indeed, it is responsible for various metabolic processes such as energy generation and storage, digestion and absorption of undigested carbohydrates, and communication with the immune system. Microbiota helps to support immune cell maturation and appropriate immune responses, regulating biological functions through diverse metabolic genes, enzymes, and biochemical pathways (Clemente et al., 2012). Additionally, the production of bioactive substances like vitamins, amino acids, and lipids is heavily dependent on gut bacteria (Hou et al., 2022). Eukaryotic organisms commonly contain miRNAs, a type of non-coding RNA that undergoes processing within the nucleus before acquiring functionality within the cytoplasm. MicroRNAs are frequently present within small, membranous vesicles such as exosomes, microvesicles, and apoptotic bodies, or they can associate with RNA-binding proteins and high-density lipoproteins. These miRNA vesicles possess varying sizes, ranging from 20 to 5000 nm in diameter (Li et al., 2019). miRNAs regulate more than 60% of human protein-coding genes, in which the human genome contains 1881 high-confidence miRNAs (Cui et all., 2016). Endogenous miRNAs are present in human plasma, urine, saliva, and body fluids, and they have been linked to various diseases including obesity, diabetes, and cancer (Li et al., 2019). Moreover, microRNAs (miRNAs) act as regulators of epigenetic mechanisms by influencing the synthesis of proteins that modify gene expression without causing DNA mutations. Epigenetic regulation plays a critical role in developing and maintaining cellular identity, function, and response to environmental stimuli. The reversible nature of epigenetic changes makes them potential targets for therapeutic interventions in various diseases (Yao et al., 2019; Ramzan et al., 2021). The miRNA-epigenetic feedback loop is regulated by DNA methylation, RNA modification, and histone modification (Yao et al., 2019). The diet could modify gene expression through miRNA regulation and its potential impact on disease development has been supported by the recent emergence of a new field called nutrimiRomics. NutrimiRomics combines health, diet, and genetics to understand how dietary elements influence gene expression, with miRNA research. Recent studies suggest that miRNAs present in diet can be absorbed by the host gastrointestinal system and modulate miRNA machinery, similar to minerals, vitamins, and micronutrients, potentially aiding in the maintenance of healthy homeostasis. The stability of miRNAs is controversial. Various studies have revealed that miRNAs are sufficiently stable throughout food processing and digestion whereas other studies widely believed that RNA could be destroyed during boiling. Nevertheless, miRNA networks play a significant role in regulating tumour metastasis-induced gut metabolites. Host intestinal cells secrete miRNAs that can regulate microbial growth and the abundance of intestinal microbiota by exchanging DNA. The gut microbiota can affect host intestinal miRNAs and modulate innate and adaptive intestinal immunities (Fan et al., 2022; Bi et al., 2020). Therefore, the role of miRNAs in regulating gene expression, modulating the gut microbiome, and their potential as a dietary intervention to improve gut health and prevent gastrointestinal diseases is an active area of research. The interaction between miRNAs and gut microbiota is crucial in regulating intestinal homeostasis, and dysbiosis of the gut microbiota is strongly correlated with a variety of intestinal diseases. Understanding the connections between gastrointestinal diseases, miRNA stability, and other

variables impacting intestinal health could potentially lead to the development of miRNA-based therapies for digestive disorders (Bi et al., 2020). Thus, this PhD project will address the controversy surrounding miRNA stability and its impact on gut microbiota and their metabolites, specifically focusing on how miRNAs regulate the gene expression of microorganisms. The results of this research will provide valuable insights into the potential of plant-derived miRNAs for gut health.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Stability of miRNAs** will be evaluated through both in vitro assessment using commercial enzymes (A1.1) and in vivo trials involving oral digestion (A1.2). This approach relies on the digestion of microRNA-containing purified extracts provided by Mirnagreen and subsequent quantification to determine its effectiveness.

A2) **Capacity of miRNAs to change the functionality of high-potential probiotics** will be tested to assess differences in fermentative profiles, phenotypes, and functionality of probiotics at different conditions using the MicroArray (PM) platform (OmnilogSystem) (A2.1) and genomics analysis (A2.2). Based on that investigation, the capability of microRNAs to enhance the production of neurotransmitters or other compounds that have health-promoting effects will be assessed.

A3) **Microbiota profile at the gut level using SHIME** will be determined to investigate the stability of microRNAs during gastrointestinal transit and their impact on gut microbial populations and cell activity. The system simulates the human gastrointestinal tract and includes five bioreactors to mimic the stomach, small intestine, and colon. The microRNA-containing extracts will be fed to the system and miRNA survivability will be evaluated through extraction and quantification methods (A3.1). During the treatment, short-chain fatty acids (SCFA), 16S rRNA and volatile compounds (VOCs) will be analyzed (A3.2).

A4) **Health-promoting properties of microRNAs at the intestinal level using Caco-2 cell** will be analyzed to assess the capacity of miRNA extracts to affect the cellular redox state (A4.1), the release of pro-inflammatory mediators, and the permeability of the human colon carcinoma Caco-2 cells (A4.2).

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***MicroRNA stability***																								
	1) Static *in-vitro* digestion																								
	2) Oral digestion (*in-vivo* trials)																								
A2)	***Effect of miRNAs on the functionality of potential probiotics***																								
	1) Evaluation of metabolic profile																								
	2) Genomic Analysis																								
A3)	***Effect on gut microbiota (SHIME)***																								
	1) Quantification of miRNAs																								
	2) 16S taxonomy and VOCs, SCFA																								
A4)	***Conducting Caco-2 cells model***																								
	1) Antiradical properties																								
	2) Anti-inflammatory properties																								
A5)	***Thesis and Paper Preparation***																								

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Innovative Technologies and Processes for Sustainable Frozen Foods

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This Ph.D. thesis aims to investigate how the quality of food products is influenced by innovative freezing technologies, eco-friendly processes, and storage systems and compare them with conventional methods.

Tecnologie e Processi innovativi per surgelati sostenibili

Questa tesi di dottorato di ricerca si propone di indagare come la qualità dei prodotti alimentari sia influenzata da tecnologie di congelamento innovative, processi ecologici e sistemi di conservazione e confrontarli con metodi convenzionali.

1. State-of-art

In recent years, there has been a growing demand for frozen foods due to their convenience, extended shelf life, and ease of transportation. However, the frozen food industry has been criticized for its high energy consumption, carbon footprint, and usage of non-renewable resources. The development of sustainable frozen food production is a complex and challenging task that requires innovative technologies optimized for the reduction of the environmental impact of this process while keeping the quality of frozen foods high. Besides its convenience, freezing and frozen storage can cause irreversible physical changes that the quality characteristics of the product such as flavor, texture, etc. may compromise thus reducing the market potential. however appropriate process conditions and pre-treatments can help prevent negative impacts (Neri et al., 2014). Studies are shown that, freezing pre-treatments, like blanching inactivates endogenous enzymes, and the use of cryoprotectants able to limit physical and structural damage the food products (Neri et al., 2020, Santerelli et al., 2019). One of the key areas of sustainable frozen food production is the reduction of energy consumption during the freezing process. Various technologies such as high-pressure, pulsed electric field- (PEF), and ultrasound-assisted freezing have been developed and combined with conventional technologies to achieve more energy-efficient and/or faster freezing. High-pressure shift freezing utilizes elevated pressures to rapidly freeze food while preserving the product's texture and minimizing ice crystal formation (Benet et al., 2004); PEF-assisted freezing applies short-duration, high-voltage electrical pulses to induce rapid freezing by forming ice crystals within the food (Mok et al., 2015); ultrasound-assisted freezing, utilizes high-frequency sound waves to accelerate freezing rates by creating localized agitation, resulting in faster and more efficient freezing while maintaining the quality of the frozen food. Vacuum cooling, microwave-assisted freezing, and cryogenic freezing can also be used for fast and efficient freezing. Another innovative approach towards sustainability is the use of renewable energy sources, such as solar and wind energy, to power the freezing equipment. In particular, solar energy has emerged as a promising renewable energy source for food freezing, especially in regions with abundant sunlight (Strielkowski et al., 2021). Solar-powered refrigeration systems offer several advantages over conventional refrigeration systems, such as reducing energy costs and carbon emissions and increasing the reliability and independence of the energy supply (Lehtola & Zahedi, 2019). Solar-powered freezers (SPF) use solar panels to convert sunlight into electricity by using photovoltaic (PV) technology. The panels made up of solar cells, capture sunlight and generate an electric current. This current, regulated by a charge controller, is stored in a battery bank as direct current (DC) electricity for later use. When the freezer needs power, an inverter converts the stored electricity (DC) from the batteries into the alternating current (AC) needed to run the freezer (Ahmed et al. 2019). The freezer's refrigeration system, including compressors and evaporators, keeps the set low temperatures in the freezer chamber. To maximize the self-consumption of PV energy, the energy requirement is adjusted to be aligned with the production of PV energy i.e., PV energy is exploited for cooling the glycol water, which assures the maintenance of cold temperatures in low-voltage cold rooms. Finally, monitoring and control systems provide real-time information on PV energy availability, enabling self-consumption and optimizing renewable energy usage. The energy efficiency of SPF is also due to the cold room configuration, specifically designed to maximize thermal insulation. However, photovoltaic generators produce energy not constantly and temperature fluctuation over time due to the nature of their power source and energy availability may occur (Ahmed et al. 2019; Lehtola & Zahedi, 2019).
So far studies on the temperature fluctuation in solar-powered systems have been carried out engineering level in terms of energy consumption and cost efficiency, while no information is reported in the scientific literature on its impact on products' quality and safety at the industrial scale. This knowledge gap needs to be fulfilled by research activities aimed to evaluate the effect of solar-powered freezers and fluctuating temperatures on the quality of frozen food products, and to the comparison with conventional processes. Limited are also the studies aimed to

evaluate the effects of freezing when coupled with innovative technologies aimed to enhance the sustainability of this process and/or the effects of freezing and frozen state of unconventional/alternative food sources (e.g., plant-based products, insects) whose consumption globally is increasing as meat-alternatives and/or the reduction of the environmental impact of food production.

2. Ph.D. Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A.1. Evaluation and optimization of the freezing process and frozen storage systems powered by photovoltaic technology for high-quality food products.
A1.1. Monitoring of temperature fluctuations in SPF chambers as a function of environmental and processing conditions will be carried out.
A.2. Evaluation of the effect of SPF freezing on selected foods. In collaboration with the company, products of different types (e.g. dairy and meats) will be selected and their main products' quality parameters defined. Instrumental and analytical methods (e.g. moisture, texture, color… water loss at thawing, sensory properties) will be optimized for the evaluation of the products' quality parameters of the products in a frozen state and their corresponding stability.
A.2.1. **Products' quality evaluation during storage in a frozen** state as a function of the process conditions will be carried out. The effect of SPF will be assessed by comparison with results obtained from samples stored in conventional freezers under similar temperature conditions.
A.3. **Application of innovative technologies on unconventional/novel food products** (e.g., plant-based products, edible insects) (secondment in NTUA, Athens, Greece).
A3.1. Innovative technologies approaches will be applied and/or combined to enhance the sustainability of the freezing process and the quality and stability of frozen food.
A.4. Writing and editing of the Ph.D. thesis, scientific papers, and oral and/or poster communications.

Table 1 *Gantt diagram for this Ph.D. thesis project.*

months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
ACTIVITIES																								
Bibliographic research																								
A1. Evaluation and optimization of the freezing process and frozen storage systems																								
Monitoring of temperature fluctuations in SPF chambers																								
A2. Evaluation of the effect of SPT freezing on selected foods																								
Products' quality evaluation during storage in a frozen																								
A3. Application of innovative technologies on unconventional/novel food products																								
Innovative technology approaches to improve the sustainability of the freezing process and the quality and stability of frozen food.																								
A4. Writing Ph.D. thesis																								

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Tomato and eggplant fruit fortification by gene editing of Glutathione S-transferase (GST) loci

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This PhD thesis research project is aimed at enhancing the nutritional quality of the fruit in tomato by CRISPR/Cas9-mediated gene editing approaches for removing inhibitory factors of GST gene expression.

Bio-fortificazione del frutto di pomodoro e melanzana mediante gene editing di loci Glutathione S-trasferasi (GST)

Questo progetto di tesi di dottorato mira ad incrementare l'espressione di geni GST mediante gene editing con tecnologia CRISPR/Cas9 volta a rimuovere la repressione operata da specifici geni regolatori e incrementare l'accumulo di antiossidanti e la qualità nutrizionale del frutto in pomodoro.

1. State-of-the-Art

Tomato (*S. lycopersicum*) is a worldwide cultivated food crop and its global yield has increased over the past decade. Its fruit contain many health-promoting compounds that have been involved in the prevention of several chronic diseases and dysfunctions (Frusciante et al., 2007. In addition to its economic and nutritional importance, tomato is an important model plant for scientific research on fruit development and quality. Since its first release in 2012, the tomato genome sequence has been widely used as a reference genome for scientific research and biotechnology assisted breeding approaches. In order to control the overall level of reactive oxygen species (ROS) and prevent cellular damage and lipid peroxidation, the plant metabolic processes deploy a plethora of bioactive compounds with antioxidant activity as defense shield against oxidative stress. The antioxidant system consists of several metabolites and enzymatic proteins such as glutathione S-transferase (GSTs). GSTs are phase II metabolic isozymes that catalyze the conjugation of the tripeptide (γ-Glu-Cys-Gly) glutathione (GSH) to a variety of substrates such as endobiotic and xenobiotic compounds for the detoxification. GSH is a key player in the plant response to the oxidative stress. In fact, GSH biosynthesis is stimulated when the cell faces stress conditions and builds up its defense capability. Also, GSH collaborates with ascorbate and NADPH in the Foyer-Halliwell–Asada cycle (Potters et al., 2002) for H_2O_2 detoxification preserving cells from damages brought about by exceeding levels of ROS. In plants, GSTs exist as a multigene superfamily and can be grouped in cytosolic, mitochondrial and microsomal. Based on their sequence plant GSTs are categorized in distinctive classes, that are tau (U), phi (F), theta (T), zeta (Z), lambda (L), dehydroascorbate reductase (DHAR), γ-subunit of the eukaryotic translation elongation factor 1B (EF1Bγ), tetrachlorohydroquinone dehalogenase (TCHQD), metaxin, Ure2p, hemerythrin (H), iota (I), microsomal prostaglandin E-synthase type 2 (mPGES-2) and glutathionyl hydroquinone reductase (GHR) (Ref.). Phi and tau are the largest plant GST classes. Within the tomato genome 90 GST genes unevenly distributed across the 13 synthetic chromosomes has been previously identified (Islam et al. 2017). Among others, chromosomes 7 and 9 harbor the highest number of GST genes, 23 and 12, respectively. Moreover, GST genes are grouped in 10 clusters and the two major tau cluster are located on chromosomes 7 and 9. Previous research carried out in our laboratory allowed us to associate in tomato GST up-regulation with fruit accumulation of phenolic compounds (Di Matteo et al., 2013). However, the use of GST genes to improve the nutritional quality of the fruit remains largely unexplored given the complexity of this gene family with a high degree of redundancy and duplication that limit the specificity of many metabolic engineering technologies and the high homeostatic strength that allows the system to react and debunk or cancel any effect of externally-induced unbalance. To functionally characterize candidate genes and breed tomato for enhanced fruit nutritional quality, genome editing technologies relay on DNA repair mechanisms that occur in cells either by non-homologous end-joining or homology directed repair systems (Steinert et al., 2016). Editing technologies, can efficiently achieve site-directed mutations of target sequences and further removal of foreign DNA by segregation in order to deal with regulatory and public acceptance issues.

2. PhD Thesis Objectives and Milestones

Our aim is to use gene editing technology that has some strengths such as: the possibility of drawing RNA specific guide to target individual loci even within families with many duplications and the possibility of drawing partially non-specific guide RNA in in order to simultaneously target genes of the same subfamily and limit the homeostasis reaction. Another innovative element of the project proposal is the possibility of pursuing metabolic alterations by editing loci miRNA to remove the inhibition on the target gene.

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Target selection** among cluster 7 and cluster 9 in order to design specific gRNAs (A1.1) and purchase PCR primers for cloning (A1.2).

A2) **Purification of vectors (A2.1) and cloning (A2.2)** of gRNAs into appropriated entry and destination vectors in order to prepare binary vectors for CRISP/Cas9 applications by GoldenBraid 3.0 technology.

A3) **Plant transformation.** Agrobacterium tumefaciens LBA4404 will be transformed with cloned binary vectors and appropriate cell cultures will be set for co-cultivation (A3.1). tomato leaf and hypocotyl explants will be co-cultivated and incubated for callus development. Callus undergo shoot regeneration and further rooting on appropriate media (A3.2). Rooted plants will be propagated in vitro and adapted to in vivo environment in an appropriated plant growth chamber.

A4) **Molecular, eco-physiological and nutritional characterization of mutants**. In order to better understand the effect of the induced knock-out, selected F2 edited plants undergo genomic DNA extraction and PCR target amplification and sequencing for validating the severe mutation. Similarly, predicted off-target mutations will be checked by sequencing. Expression analysis of GSTs sharing high sequence similarity will be performed by TaqMan qPCR. Similarly, the effect on the regulation of genes involved into the response to the antioxidant stress and antioxidant biosynthesis will be analysed by qPCR (A4.1). Plants grow in an appropriate greenhouse will be characterized for leaf photo-assimilation efficiency, response to antioxidant stress, fruit set-up and yield (A4.2). Fruits will be analysed for antioxidant profiling (A4.3).

A5) **Recombination and T-DNA elimination (A4).** In order to eliminate the engineered T-DNA, edited plants will be self-pollinated and F2 segregants will be selected for keeping the edited alleles and missing the T-DNA insertion by using appropriate TaqMan molecular markers.

A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Target selection***																								
	1) Design of gRNAs																								
	2) Design and purchasing of PCR primers																								
A2)	***Purification of vectors and cloning***																								
	1) Purification of vectors																								
	2) Cloning																								
A3)	***Plant transformation***																								
	1) Preparation of *A. tumefaciens* cultures																								
	2) Preparation of explants, co-cultivation, callus development and regeneration																								
A4)	***Charachterization***																								
	1) Molecular characterization																								
	2) Ecophysiological characterization																								
	3) Nutritional phenotyping																								
A5)	***Ricombination and T-DNA elimination***																								
A6)	***Thesis and Paper Preparation***																								

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Fluorinated organic pollutant assessment in agri-food-based matrices in different environmental scenarios

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Among the persistent organic pollutants are perfluoroalkyl substances, known as PFAS. The project aims to assess the content of targeted and non-targeted PFASs in agri-food products from areas of different anthropogenic impact, aimed at determining current levels, sources of contamination, their trend over time, and possible correlation with other pollutants.

Valutazione degli inquinanti organici fluorurati in matrici agroalimentari in diversi scenari ambientali

Fra gli inquinanti organici persistenti vi sono le sostanze perfluoroalchiliche, note come PFAS. Il progetto ha come obiettivo la valutazione del contenuto di PFAS targeted e non-targeted in prodotti agroalimentari provenienti da zone a diverso impatto antropico, volto alla determinazione: degli attuali livelli, delle fonti di contaminazione, del loro andamento nel tempo e dell'eventuale correlazione con altri inquinanti.

1. State-of-the-Art

The exponential industrial and scientific development of the last century has led to the production and use of many chemicals that have since been found to be harmful or potentially harmful to the environment and human health. Among the toxic substances classified as Persistent Organic Pollutants (POPs) are poly and perfluorinated compounds (PFAS), which are extremely important both from a toxicological and environmental aspect. These molecules are characterized by carbon chains containing fluorine atoms partially or completely replacing hydrogen atoms. They are found to be particularly recalcitrant and persistent in the environment because of the strong bond between carbon and fluorine, which gives them considerable chemical stability. Their use in many industrial fields is favored by the different chemical/physical characteristics given by the different substituents that can be linked to the alkyl chain. PFASs can be grouped into three macro-categories: i) perfluoroalkyl acid (PFAA) and its precursors, used for fire-fighting foams and to protect surfaces of textiles, clothing, leather, carpets and paper as well as for the production of fluorotelomers; ii) fluorotelomers, used for the production of plastics and rubbers and as water-repellent agents in paints; and iii) hydrofluorocarbons, used for the production of heat transfer fluids and detergents (Glüge *et al*., 2020). The scientific community's concern about such molecules is due to their identification as endocrine disruptors involving both female fertility problems-polycystic ovary syndrome and interference with normal mechanisms of pre- and postnatal development. Scientific studies have shown increased production of ROS and a possible association between their presence in the human body and cancers of the liver, testes, and pancreas. Some molecules including perfluorooctanoic acid (PFOA) have been shown to be harmful to the molecular structure of DNA by increasing the probability of genetic mutations (Bonato *et al*., 2020). In Italy, major concern was raised by the analysis promoted in 2013 by the National Research Council (CNR) and the Ministry of the Environment and Protection of Land and Sea (MATTM) in the Veneto region, where large-scale PFAA contamination was revealed in drinking water (Bonato *et al*., 2020). Furthermore, another substantial source of human intake of PFAS resides in food. The foods most susceptible to PFAS contamination are those of a protein nature, in fact in contrast to other POPs, PFAS preferentially accumulate in protein-rich (not lipid-rich) tissues with subsequent possible biomagnification. Thus fish products, wild game and edible animal offal represent one of the major sources of PFAS intake by humans, especially considering perfluorooctanesulfonic acid (PFOS) (Authority EFS, 2011). The soil from which agri-food commodities are grown can be contaminated with PFASs either from irrigation water or from the use of sewage sludge used as a soil conditioner, which itself has contamination. It is the organic fraction of soil that promotes the accumulation of PFAS within the soil from which it subsequently migrates within plants. The breakdown within the plant is not homogeneous for all PFASs; the compounds that accumulate most in leaves and fruits are the short-chain C4-C6 compounds, compounds that nowadays are used to replace PFOA and PFOS. In contrast, the long-chain ones are more concentrated in roots (Ghisi *et al*., 2018). Food packaging and cooking utensils can also be a source of PFASs in food given the use of these substances in lipophobic coatings and nonstick materials. Just as with other pollutants, there is a problem with PFASs migrating from the packaging or utensil to the food. The results of a recent study showed that it is the short-chain PFASs that are most affected by release into food. Migration is influenced by a number of factors such as: repeated reuse; exposure time; temperature; lipid, salt and pH content of the food (Ramírez Carnero *et al*., 2021). For this reason, the European Food Safety Authority (EFSA) set a group safety threshold for PFAS in 2020 regarding the limit for tolerable weekly intake (TWI) of 4.4 ng kg^{-1} body weight (EFSA CONTAM Panel, 2020).

Moreover, the EU has enacted Directive 2020/2184, concerning the quality of water intended for human consumption, setting limits for drinking water at 0.50 μg L^{-1} concerning the content of total PFAS, granting a transitional period until January 12, 2026, and Regulation 2022/2388 setting PFAS limits for certain foods. Nowadays, we speak of total PFASs because since 1940, many structural or isomeric variants of the original PFASs have been developed so the analysis of such molecules has shifted from a targeted to a non-targeted approach. The analysis of non-targeted molecules allows a comprehensive assessment of the content of PFASs that may be present in the analyzed matrix, and only in this way is it possible to make comprehensive estimates of the presence and abundance of these compounds in the environment-and thus in agri-food products and of their variation over time (Gonzalez de Vega *et al*., 2021). While food contamination for PFAS is often due to their use in other fields, foodstuffs are still affected by other chemical contaminants such as pesticides. These substances can be found in food as residues from treatments carried out on crops in order to increase production yield and meet food demand. They are a concern for human health because they can lead from simple headaches to more serious diseases such as cancer, as well as for the entire environmental ecosystem, and it is important to monitor their presence in foods to safeguard the health of consumers (de O. Gomes *et al*., 2020).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Study of analytical method** starting with the choice of target molecules (A1.1) to develop an analytical method based on scientific knowledge and new analytical approaches (A1.2).
A2) **Sampling:** selection of sampling areas based on anthropogenic impact (A2.1) and sampling of agri-food products (A2.2).
A3) **Laboratory work** to analyze samples (A3.1) and check for chemical contaminants by data processing (A3.2).
A4) **Data analysis** to study sample clustering and correlations between molecules (A4.1) and to study sources of contamination (A4.2)**.**
A5) **Writing and Editing** of scientific reports and papers and the PhD thesis.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Study of analytical method***																								
	1) Choice of target molecules																								
	2) Development of an analytical method																								
A2)	***Sampling***																								
	1) Choice of sampling areas																								
	2) Sampling of agri-food products																								
A3)	***Laboratory work***																								
	1) Sample analysis																								
	2) Data processing																								
A4)	***Data analysis***																								
	1) Statistical analysis																								
	2) Study of sources of contamination																								
A5)	***Thesis and Paper Preparation***																								

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Characteristics and role of food labeling to favour the transition towards sustainable and healthy diets: nutritional, health and regulatory aspects

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This PhD thesis research project aims to investigate how labelling can help educate consumers and improve their eating behaviour by using the information provided on the Front-Of-Pack, to strengthen their power of choice in favour of healthier and more sustainable diets.

Caratteristiche e ruolo dell'etichettatura alimentare nel favorire la transizione verso diete sane e sostenibili: aspetti nutrizionali, salutistici e normativi

Questo progetto di ricerca di tesi di dottorato ha lo scopo di indagare come l'etichettatura possa contribuire a educare i consumatori e migliorare il loro comportamento alimentare, utilizzando le informazioni fornite sul Front-of-Pack per rafforzare il loro potere di scelta a favore di diete più sane e più sostenibili.

1. State-of-the-Art

The food system is a complex picture in which food production and nutrition are strictly linked (FAO, 2018). Climate change and non-communicable diseases (NCDs) are also associated, representing the big threats of the 21st century (FAO and WHO, 2019). A sustainable food system stands at the heart of the United Nations' Sustainable Development Goals (SDGs) signed by 193 countries belonging to the United Nations, in 2015, and consecutively implemented by the Sustainable Healthy Diets (SHD) Guiding Principles document, published in 2019 as a call for major transformations in food systems to end hunger, achieve food security, and improve sustainable nutrition. Among all the stakeholders, the European Commission signed in 2019 the Green Deal, a set of policy initiatives proposed with the overall goal of achieving climate neutrality in Europe by 2050. One of the operative documents in it, titled The Farm to Fork Strategy, recognizes, among all the actions proposed, the use of food policy documents, such as food based dietary guidelines (FBDG), and the use of labels, as two mighty tools to empower citizens in favour of healthier and more sustainable diets made of a variety of foods that needs to be consumed in adequate portions and frequencies of consumption. If on one hand the criticality of food policies is the lack of heterogeneity in the information provided (e.g., qualitative, or quantitative information relative to frequency of consumption and serving sizes); the concern about labelling is that the systems for classifying food products to adequately predict lower all-cause mortality that have been proposed as front-of-pack (FOP) nutritional labels (Carruba et al., 2021) are many and different. One of the most debated is the NOVA classification that classifies foods in four groups: unprocessed and minimally processed foods (group 1), processed culinary ingredients (group 2), processed foods (group 3), and ultra-processed foods (UPF) (group 4) (Monteiro, 2019); according to the extent and purpose of food processing, rather than in terms of nutrients. Although the efforts and advantages that this system represent for public health, some FOP labels, included the NOVA based one, risk hampering consumers awareness of nutritional food quality, and in the specific case of NOVA, the lack of strong scientific evidence is the main concern. Since there is preponderant evidence indicating that are the ingredients of ultra-processed foods those moderating people's health, and not the processing tout court, it is premature to push for the use of a classification system about the role of UPF in diets, before the verification of causality and plausible mechanisms of action (Valicente et al., 2023).
Thus, this PhD thesis project will be directed to investigate how food labelling information can contribute to acquisition of knowledge by the consumer and promote healthier eating behaviour without demonizing any kind of food item but to rather encouraging conscious dietary choices.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Literature revisions to cover all the gaps in knowledge. A first literature revision about portion size pack declaration effects on human diet quality (A1.1). Review of the inclusion of food labels in the national and

international food policy documents, for example Food based Dietary Guidelines. Indications on portion size and frequency of consumption present in FBDG will be investigated and divided into qualitative and quantitative indications. Both qualitative and quantitative indications will be recorded and compared in terms of target population considered, food groups classification, unit of measure used, portion sizes quantification for each food group, and frequency of consumption (A1.2)

A2) Comparison of the content of nutrients and potentially harmful compounds in ultra-processed and minimally processed foods (UPF project). This part of the project will be carried out in the Wageningen University, in the Food Quality and Design department. Industrial products will be compared with laboratory-produced analogues resembling the original industrially produced one, utilizing, when possible, the same ingredients exploited at the factory level. Preliminary modelling of the project and ingredients choose (A2.1). Performance of the project and realization of the designated foods. Macronutrient composition, energy density, and Maillard reaction's products (e.g., Acrylamide) will be investigated and compared (A2.2).

A3) **Design of human intervention studies and generalized experimental procedure**. A 3-arms randomized trial will be settled. Participants will be randomly assigned to take home a regular pack of foods (e.g., products with different degrees of processing), a regular pack of foods carrying a label about serving information, or the same number of food units of a regular bag, singularly packed in recommended servings and carrying the serving label. (Recommended quantities will be aligned with those of the Italian Dietary Guidelines for a reference intake of 2000 kcals). Subjects will be asked to fill a food diary with the aim of investigating the role of the information provided on diet quality and eating behaviour (A3.1)

A4) **Human intervention Study data analysis** (A4.1 and 4.2)

A5) **Thesis preparation**

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Literature revisions***																								
A1.1	Review on the role of portion size declaration effects on human diet quality																								
A1.2	Review on the inclusion of food labels in the national and international food policy documents																								
A2)	***UPF project-WUR***																								
	UPF Project Modelling																								
	UPF project Performance																								
A3)	***Human Intervention Study***																								
	Modelling																								
	Performing the Intervention																								
A4)	***Human Intervention Data Analysis***																								
	RCT																								
	RCT data analysis																								
A5)	Thesis and paper preparation																								

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Grape related yeasts as source for designing specific "synthetics microbiota" to be used for wine fermentations.

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This PhD project aims to highlight the importance of grape microflora in increasing the diversity and improving quality of wines from different *terroirs*. The use of commercial starter cultures (mainly *S. cerevisiae*) reduces the potential of the native microbiota to contribute to the terroir effect. The project involves the development of starter culture design protocol (synthetic microbiota) made up from different selected species from specific *terroirs*. The synthetic microbiota could bring improvements from a sustainability point of view by exploiting the individual characteristics of each selected strains to achieve greater complexity, stability and natural protection of wine.

I lieviti dell'uva come fonte per la progettazione di specifici "microbiota sintetici" da utilizzare per le fermentazioni vinarie.

Questo progetto di dottorato mira a evidenziare l'importanza della microflora dell'uva nell'aumentare la diversità e migliorare la qualità dei vini provenienti da diversi *terroirs*. L'uso di colture starter commerciali (principalmente *S. cerevisiae*) riduce il potenziale del microbiota nativo di contribuire all'effetto terroir. Il progetto prevede lo sviluppo di un protocollo di progettazione di colture starter (microbiota sintetico) costituito da diverse specie selezionate provenienti da specifici *terroirs*. I microbiota sintetici potrebbero portare miglioramenti dal punto di vista della sostenibilità sfruttando le caratteristiche individuali di ciascun ceppo selezionato al fine di ottenere una maggiore complessità, stabilità e protezione naturale del vino.

1. State-of-the-Art

In recent decades, the biodiversity of the microflora present on the grape surface has been extensively studied, and the main genera of yeasts and fungi present are well known (Castrillo *et al*., 2019). The three most frequently isolated species on the berries surface are *Hanseniaspora uvarum*, *Metschnikowia pulcherrima* and *Starmerella bacillaris*, which also appear to be the dominant species in the must. However, also considering the application of culture-independent (meta-taxonomic) approaches, the real diversity is much higher: 50 species of yeast, belonging to 22 different genera, including *Auerobasidium, Auriculibuller, Brettanomyces, Bulleromyces, Candida, Cryptococcus , Debaryomyces, Hanseniaspora, Issatchenka, Kluyveromyces, Lipomyces, Metschnikowia, Pichia, Rhodosporidium, Rhodotorula, Saccharomyces, Sporidiobolus, Sporobolomyces, Torulaspora, Yarrowia, Zygoascus, and Zygosaccharomyces were identified* (Bokulich and Mills, 2013; De Filippis *et al*., 2017). The diversity and abundance of yeast populations is linked to various factors, in addition to the degree of ripeness of the grapes and the relative variety, the pedoclimate of a specific area, the phytosanitary state of the grapes, agronomic practices and human activities (Wei *et al*., 2022). Currently, there is a greater demand, especially for organic wine producers, to make the best use of the microbiological diversity present in their vineyards. By contrast, the improper and uncontrolled use of non-*Saccharomyces* species could lead to problems both during the fermentation phases and for the final wine quality (spoilage). However, a careful selection of these yeasts could lead to greater complexity, variability and uniqueness of the final product. Although some strains of these species do not have excellent fermentative performances, they can help to improve the sensory attributes of wine thanks to the production of extracellular enzymes and secondary metabolites such as esters, higher alcohols, acids and glycerol. The challenge nowadays is to identify oenologically interesting non-*Saccharomyces* yeasts from different *terroirs*, carrying out an analysis of the metabolic activities of single strains and subsequently evaluating the interactions with other species/strains, then proceeding towards the creation of a synthetic microbiota that simulates spontaneous fermentation. The central theme that climate change has highlighted in enology is certainly the increase in sugars in grapes and therefore a greater quantity of potential alcohol in the final wine, therefore also a lower presence of fixed acids, in fact the use of yeasts non-*Saccharomyces* in co-inoculation, could be an alternative to have a lower ethanol yield, in relation to the quantity of sugars present in the starting must (Castrillo *et al*., 2022).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:
A1) **Evaluation of the microbial diversity** of grapes, musts and wines from different *terroirs* (meta-taxonomic

analysis).
A2) **Isolation** (culturomic analysis) molecular (identification, biotyping and genomics) and technological characterization of yeasts strains. Genomics analyses will be performed during a laboratory experience abroad.
A3**) Bottom-up,** metabolic activities of individual strains and their interactions.
A4) **Top-down,** design of the synthetic microbiota by lab-scale experiments and use by cellar-scale trials.
A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months		*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*
A1)	***Evaluation of the microbial diversity***																								
	Sampling and DNA isolation																								
	Meta-taxonomic analysis																								
A2)	***Culturomic analysis***																								
	Yeast isolation																								
	Identification, biotyping and genomics																								
	Technological characterization																								
A3)	***Bottom-up***																								
	Metabolic activities of individual strains																								
	Interactions between strains																								
A4)	***Top-down***																								
	Lab-scale trials																								
	Cellar-scale trials																								
A5)	***Thesis and Paper Preparation***																								

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Microbiome Mapping in Meat Food Chain from Farm-to-Fork

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This Ph.D. research project develops in collaboration with Dawn Meats Group, a main meat producer and processor in Ireland. The project aims to evaluate the main microbial players in meat spoilage, with a focus on identifying these microbial spoilers as well as their possible routes of contamination throughout the meat production chain (i.e., at the farm, slaughterhouses, processing and packaging facilities, transport, retailer/market, up to the final consumer) using culture-independent shotgun metagenomics. Metagenomics will allow to investigate the presence of microbial genes associated with potential hazards (e.g., antibiotic resistance, virulence factors, and toxin production) and spoilage-related activities (e.g., proteolysis, production of volatile compounds and off-odours, slime, biofilm production). Finally, novel preservation technologies (PAW, plasma activated water) will be tested to improve beef safety and shelf life.

Mappatura del microbioma lungo la catena di produzione della carne bovina "from farm-to-fork"

Il progetto di ricerca verrà sviluppato in collaborazione con Dawn Meats Group, uno dei principali produttori di carne in Irlanda. Il progetto mira a valutare i principali agenti microbici responsabili del deterioramento della carne, con particolare attenzione all'identificazione delle loro possibili vie di contaminazione lungo tutta la catena di produzione della carne (ovvero, dall'allevamento, al macello, fino agli impianti di lavorazione e confezionamento, e lungo il trasporto fino al consumatore finale) utilizzando un approccio di metagenomica shotgun, che consentirà di indagare la presenza di geni microbici associati a potenziali pericoli per la salute (ad es. resistenza agli antibiotici, fattori di virulenza e produzione di tossine) e attività correlate al deterioramento (ad es. proteolisi, produzione di composti volatili dall'odore sgradevole, produzione di biofilm). Infine, verrà testato l'utilizzo dell'acqua attivata al plasma per prolungare la sicurezza e la shelf-life della carne.

1. State-of-the-Art

Meat consumption has been steadily increasing worldwide due to its nutritional value and delicious flavours, with an estimated 346 million metric tons consumed in 2021 alone (FAO, 2021). However, meat and meat products are extremely vulnerable to the colonization and development of a wide range of microorganisms (Cauchie *et al.,* 2020). Meat spoilage is a major issue around the world, accounting for up to 20% of total meat production losses (Karwowska *et al.,* 2021). In addition, several pathogens may develop in raw beef, representing a health concern (e.g., *Listeria monocytogenes, Salmonella* spp., pathogenic *Escherichia coli*).
The advent of next-generation sequencing revolutionized the collection of massive amounts of data from microbial ecosystems, overcoming the limitations of culture-based and PCR-based methods (Almeida and De Martinis, 2019).

2. PhD Thesis Objectives and Milestones

The goal of this research project is to map the microbiome of the entire beef chain using metagenomics, providing insights into how to improve microbiome monitoring and management throughout the beef supply chain.
The project can be divided into the following activities according to the Gantt diagram reported in Table 1:

A1) Literature review on the role of microbiome on fresh beef spoilage: A literature review will be carried out to identify the main contaminations routes in order to effectively plan the sampling activities.

A2) Analysis of contamination routes in raw beef chain: The primary goal of this study is to identify and characterize the microbes involved in meat spoilage, along with identifying potential routes of microbial contamination, such as the processing environment, equipment, or personnel (i.e., from farm to end retailer) in the entire meat production chain. This activity will be in collaboration with the Dawn Meats Group in Ireland, that own 6 different slaughterhouses and processing facilities across Ireland, as well as several farms (Figure 1). We plan to sample 5 different animals in each farm and follow the same animals along the processing chain, The sampling will include environmental swabs taken on surfaces, tools and equipment in the different facilities, as well as raw beef before and after the maturation, after the slicing and packaging and during the shelf life, for a total of around 300 samples. The sampling will be carried out twice, during summer (August-September 2023, when the animals are grazing) and in winter (January-February 2024), when they are in the stalls. Samples will be sequenced using a shotgun metagenomics approach. We will be able to identify the routes of transmission of the microbiome through the meat production chain, from farm to table, and determining its ability to survive in various environmental conditions. In addition, we will figure

out how these microbiomes cause meat spoilage, identifying the microbial genes and metabolic pathways that contribute to meat spoilage, such as the production of off-flavors, protein breakdown, meat discoloration, as well as genes related to potentially harmful activities (toxin production, virulence factors).

A3) **Exploring new strategies for meat prevention and preservation** in a second part of the study, we will focus to develop and test novel strategies for controlling meat spoilage. During a secondment at the University of Leon (Spain), the effect of Plasma Activated Water on beef contamination will be evaluated, to define a potential use for carcass washing.

A4) **Thesis and Publications writeup:** We will share our findings with the scientific community as well as the cooperating company. Research findings will be published in peer-reviewed scientific journals.

A5) **Research dissemination:** key findings will be presented at national and international scientific conferences and meetings.

Table 1 Gantt diagram for this PhD thesis project.

S. No	Activities	Time Frame (3 Years)								
		1st January 2023 – 31st December 2025								
		2023			2024			2025		
A1)	**Literature review on the role of microbiome on fresh beef spoilage**	**Q1**	**Q2**	**Q3**	**Q1**	**Q2**	**Q3**	**Q1**	**Q2**	**Q3**
A2)	**Analysis of contamination routes in raw beef chain**									
	1) Microbiome Sampling from different locations and in different seasons (2 times)									
	2) DNA extraction and shotgun metagenome sequencing									
	3) Bioinformatics data analysis									
A3)	**Exploring new strategies for meat prevention and preservation**									
	1) Evaluating Plasma Activated Water (Secondment at Univ. Leon, Spain)									
A4)	**Thesis and Publications writeup**									
A5)	**Research Dissemination**									

Note: Q: Quadrimester

Figure 1. The sampling plan discussed with the Dawn Meat Group, Ireland, and the different facilities that will be sampled.

DAWN MEAT IRELAND:

Flow chart for sampling to investigate identified interest points.
Primary route will be:

1. Farm
2. Slaughter Plant
3. Cutting Plant (could be same site or different)
4. Retail Packing Plant
5. Distribution (lorry & depot)
6. Retail stores

3. References:

1. Almeida, O.G.G.D. and De Martinis, E.C.P., 2019. Relating next-generation sequencing and bioinformatics concepts to routine microbiological testing. *Electron. J. Gen. Med*, ***16***.
2. Cauchie, E., Delhalle, L., Taminiau, B., Tahiri, A., Korsak, N., Burteau, S., Fall, P.A., Farnir, F., Baré, G. and Daube, G., 2020. Assessment of spoilage bacterial communities in food wrap and modified atmospheres-packed minced pork meat samples by 16S rDNA metagenetic analysis. *Frontiers in microbiology*, ***10***, p.3074.
3. Food and Agriculture Organization of the United Nations (FAO), 2021. Meat Market Review: Emerging Trends and Outlook.
4. Karwowska, M., Łaba, S. and Szczepański, K., 2021. Food loss and waste in meat sector—why the consumption stage generates the most losses?. *Sustainability*, ***13***(11), p.6227.

Low-cost and Novel Sensors for Fruit Maturity Assessment Along the Whole Quality Chain

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This Ph.D. project aims to develop non-destructive method for estimating apple maturation during pre-harvest and post-harvest using Electrical impedance spectroscopy (EIS) and optical techniques. It focuses on developing a portable impedance analyser, and a machine learning-based classification method for fruit ripening discrimination. Machine learning approaches will be used to develop models for discriminating apple ripening status. Data analysis of optical spectroscopy and bioimpedance using machine learning will be optimized, tested, and compared to provide efficient real-time estimation of apple ripeness at pre-harvest and post-harvest stage. This model objective is to empower local companies to optimize apple production, storage, and processing for improved efficiency and sustainability.

Sensori innovativi ea basso costo per la valutazione della maturità della frutta lungo l'intera catena della qualità

Questo dottorato di ricerca Il progetto mira a sviluppare un metodo non distruttivo per stimare la maturazione delle mele durante la pre-raccolta e la post-raccolta utilizzando la spettroscopia di impedenza elettrica (EIS) e tecniche ottiche. Si concentra sullo sviluppo di un analizzatore di impedenza portatile e di un metodo di classificazione basato sull'apprendimento automatico per la discriminazione della maturazione dei frutti. Verranno utilizzati approcci di apprendimento automatico per sviluppare modelli per discriminare lo stato di maturazione delle mele. L'analisi dei dati della spettroscopia ottica e della bioimpedenza utilizzando l'apprendimento automatico sarà ottimizzata, testata e confrontata per fornire un'efficiente stima in tempo reale della maturazione delle mele nella fase pre-raccolta e post-raccolta. L'obiettivo di questo modello è consentire alle aziende locali di ottimizzare la produzione, lo stoccaggio e la lavorazione delle mele per una maggiore efficienza e sostenibilità.

1. State-of-the-Art

Apples are among the most popular consumed fruits in the world. The European Union is one of the biggest apple producers, after China. With a share of 19.2% Italy is the second European producer after Poland (25%) (https://ec.europa.eu/eurostat (accessed on 18 July 2021). The total apple production of EU was 12.2 million tons in 2020 (https://www.statista.com/statistics/577753/apples-production-volumeeuropean-union/, accessed 18 July 2021)(DeMeyer, 2014). To maintain the check on quality and ripeness assessment of apple throughout all stages of production is crucial for meeting consumer demands, reducing food waste and improve the profit margin of producers. Food and Agriculture Organization of the United Nations (FAO) refers to fruit waste as "the decrease in the quantity or quality of fruit resulting from decisions and actions by retailers, food service providers and consumers" (Angelo Zanella and Sadar Nadja, 2021). At each stage of the fruit supply chain, from cultivation to final consumption, fruit quality assessment is integral to reducing financial losses. The post-harvest sorting of fruit is particularly essential, with both internal and external quality attributes and defects considered in identifying damages caused by mishandling - mostly related to mechanical (pressure, impact) and temperature (freezing, thermal shocks) issues (Jha *et al.*, 2019). A range of techniques exist to assess fruit quality before harvest and post-harvest, with correct determination playing a key role in the entire supply chain management. For the quality of apples following parameters are included; firmness, texture, core color, internal flash color, soluble solids concentration (SSC), starch, soluble sugars concentration, chlorophyll content for maturity of fruit, and the internal disorder (Li and Thomas, 2014). However, dealing with overripe fruits during postharvest is a challenging task that results in negative impacts on their storage and marketing (Atkinson *et al.*, 2014). These all parameters are analysed by conventional destructive techniques and non-destructive techniques. At present, destructive methods are widely investigated, their qualitative and quantitative measurements are very precise and accurate, and high sensitivity, for instance, dynamometer for firmness, refractometer for sugar concentration and starch index for ripening. However, these techniques also have many limitations; expensive, long wet lab based experimental set up, slow, time-consuming, and require sample preparation, thus they cannot be used for large-scale measurement, for instance in field and in food sheds, and storage houses. This method also creates damage in the sample which is not ideal for industrial purposes (Srivastava and Sadistap, 2018). To overcome the wastage of food and for environment safety, non-destructive technologies are recently developed and attract market attention because it allows repeated quality measures on the same fruits without damaging it. With increasing demands for real-time detection of fruit quality at the industrial level, it is necessary to develop non-destructive and non-contact detection

systems (Nicolaï *et al.*, 2007).The most common used non-destructive analysis are optical techniques, based on the interaction of the light with the sample under test, include colorimetry (Reid, 2002), visible imaging (Vanoli and Buccheri, 2012), visible and near-infrared (VIS-NIR) spectroscopy (Cortés *et al.*, 2019), hyperspectral and multispectral imaging (Qin *et al.*, 2013). However, the most common use NIR technique for fruit quality has some limitation too; 1) Low sensitivity, 2) wavelength overlap, 3) Not able to provide the full image of internal physiology of fruit, 4) Penetration of light is very few milli meter, 5) Not able to identify internal disorder and internal browning of fruit directly, 5) NIR spectra of fruits is dominated by water absorption bands. On the other hand, the interaction of an electric field with the fruit provides a new approach for fruit quality measurement. In this context, Electrical Impedance Spectroscopy (EIS) represents a low-cost and competitive approach compared to conventional optical methods (Ibba *et al.*, 2018). Electrical impedance spectroscopy (EIS) has shown promise as a suitable method for detecting changes in fruit physio-chemical properties(Rivola *et al.*, 2021). However, its potential in the context of fruit quality control is limited by technical constraints such as the need for bulky and expensive instrumentation, non-optimized electrode systems, and a lack of data analysis methods. Thus this Ph.D. project aims to address these issues by combining bio-impedance measurement with optical techniques like Near-Infrared spectroscopy to develop a more precise comparative model. While NIR is commonly used for fruit quality analysis, it lacks real-time assessment of internal physiology and browning (Mohsen *et al.*, 2021). This study aims to resolve this limitation by incorporating non-destructive techniques to improve precision and accuracy in evaluating fruit quality.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) **Literature Review** Current progress in utilizing EIS and optical spectroscopy for managing fruit quality and maturity parameters.

A2) **EIS and other non-destructive spectroscopic analysis** To enhance the connection between electrical and biological data, there will be efforts to correlate the bioimpedance data with the physiological changes in fruits and create new equivalent circuit models that provide a more accurate representation of the interaction between fruit quality and bioimpedance.

A3) **Comparative statistical data analysis for efficient bioimpedence model** To advance the data analysis of bioimpedance data and NIR data then its correlation with the maturity of fruit. To develop a comprehensive data analysis pipeline, including the data acquisition, reduction, transformation, and correlation, to achieve (i) a flexible platform to follow during future studies in the field and (ii) the first prediction and classification models of apple aging evolution starting from bioimpedance data.

A4) **Application of the comparative model to pre-harvest and post- harvest fruit samples** To design and validate new, affordable, and adaptable portable impedance analyzers that are specially intended for the purpose of fruit aging monitoring throughout the entire supply chain to bridge the gap between this comparative bioimpedence measuring model and its practical application in the field.

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Literature Review***																								
A2)	***EIS and other non-destructive spectroscopic analysis***																								
	1) EIS at pre and post-harvest																								
	2) Optical analysis for different quality parameters																								
A3)	***Comparative statistical data analysis for efficient bioimpedence model***																								
	1) Correlation with other analytical analysis tool																								
	2) Best fit model Prediction																								
A4)	***Application of the comparative model to pre-harvest and post-harvest fruit samples***																								
	1) Validation of comparative model																								
	2) Application on samples																								
A5)	***Thesis and Paper Preparation***																								

Sustainability, innovation and environmental impact of spray packaging applied to food and nutraceutical products: development of innovative sprayable solutions and study of their application in bakery and confectionery.

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This PhD thesis research project is aimed at setting up an experimental procedure for the identification and the development of different formulations (such as mousses, fresh doughs, dressings, and nutraceuticals) that can be packaged in aerosol spray cans used in confectionery and bakery. They will be first produced through pilot plants, then rheologically and technologically characterized; the process will be finally validated in order to be applied on an industrial scale.

Sostenibilità, innovazione e gestione dell'impatto ambientale del confezionamento spray applicato ai prodotti alimentari e nutraceutici: sviluppo di soluzioni spray innovative e studio della loro applicazione per la produzione di prodotti da forno e prodotti dolciari.

Questo progetto di tesi di dottorato mira a mettere a punto un procedimento sperimentale per l'individuazione e lo sviluppo di diverse formulazioni (quali mousse, impasti freschi, preparazioni e prodotti nutraceutici) che possano essere confezionate in bombolette spray successivamente impiegate nelle filiere dei prodotti dolciari e da forno. Queste verranno prodotte attraverso impianti pilota, caratterizzate dal punto di vista reologico e tecnologico, e il processo verrà poi validato in modo da poter essere applicato anche su scala industriale.

1. State-of-the-Art

Nowadays, industries of bakery and confectionery products are investing a lot in innovation and sustainability. This is mainly due to the new consumers' request and to the focus on the reduction of transformation impact on the environment. For these reasons, the development of innovative and more sustainable products is fundamental both for academia and industry. Innovation in bakery and confectionery can be applied in different ways (Martínez-Monzó et al., 2013). Table 1 lists the latest innovations registered in the different fields of bakery and pastry.

Table 1 Main innovations applied on bakery and confectionery (Martínez-Monzó et al., 2013).

Drivers	Innovations applied	Examples of applications
Health	Functional Ingredients	Probiotics or prebiotics addition
	Energy & Satiety	Integration with fibers, antioxidants, vitamins, proteins
	Gluten & Allergen free	Use of protein isolates, or fibers
Convenience	Smaller portions	Introduction of new formats (e.g. muffins)
	Local and Seasonal	Regional and ethnic tastes
Pleasure	Specialties	Artisan products
	Fashion flavors	Salty/sweet, sweet/spicy

The reformulation and the provision of an added-value to a product can be very useful when innovating it; but the packaging also represent an important factor that can be considered. Today's consumers are aware of environmental sustainability and the choice of the correct type of packaging is critical in trying to make it more sustainable by maintaining its preservatives properties (Mitelut et al., 2021). For these reasons, I would like to focus my doctoral project on the development of some innovative and added-value recipes that can be applied in bakery and confectionery investigating also the possibility of using a new type of pack: a sprayable can, in order to create an easy use product with an easy storage (already used for whipped cream and drugs). Aerosol products represents a good packaging solution: they are versatile, easy to use, clean and efficient; moreover, they are made of completely recyclable material, they can guarantee longer storage times without the use of cold chain, they can reduce the chances of contaminations, and they contribute to waste reduction (for no unused product to be disposed of). The initial approach of this research project will involve the application of this new packaging technique to some enriched food preparations (such as glazers or mousses) and, only later, focus the attention on cake batters (due to the fact that sponge cakes are the most popular and consumed within baked goods) (Rodríguez-García et al., 2013). This kind of samples could also have an easy application to a sprayable packaging, giving them more stability and an easier way to use. This packaging solution has already been studied for pancakes using a siphon loaded with pressurized gas (Lostie et al., 2002), so it might be easily adapted to a different type of batter (Principato et al., 2021). The samples will be analysed in order to define their microbiological and physical

stability, also considering their rheological behaviour; a shelf-life study should be also conducted to understand the stability in the long term and the effect of the packaging on the samples.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) **Research:** a deeper research focused on both the scientific results reached until now regarding bakery, confectionary, spray gases, and technique (A1.1); associated with a market research in order to understand what are the products already present on the market and what can be developed as an alternative (A1.2).

A2) **Semi-finished sprayable base preparations used in bakery and confectionery:** some base preparations already used in bakery and confectionery will be characterized and specifically modified in their composition in order to make them sprayable. The samples will be evaluated to understand their microbiological stability and safety (A2.1); and characterized in terms of rheology to have more information about their structure (A2.2). Formulations will be also subjected to a shelf-life study (A2.3), and finally some trials will be done applying them on some bakery goods (A2.4) in order to evaluate if the sprayable form can be comparable to the traditional one (from the technological and sensory point of view).

A3) **Development of a completely new sprayable product:** use of a pilot plant for the development of different kind of mousses and cake batters that will be packed into a sprayable can, also integrated with nutraceutical and functional compounds (A3.1). The samples will be first monitored to guarantee their microbiological stability (A3.2), and then analysed from the rheology point of view to obtain information related to their viscosity and stability (A3.3). A shelf-life study will be conducted to verify their long-term stability (A3.4). Finally, the final obtained product (the mousse or the baked cake) will be physically and sensory characterized (A3.5).

A4) **Scaling up at an industrial level:** once defined the most performing preparations and sprayable products, their formulations will be optimized in order to standardize the process in a pilot plant scale (A4.1); then, they will be scaled up at an industrial level with the contribution of a Company specialized in aerosol products production (A4.2).

A5) **Writing and Editing** of the PhD thesis, scientific papers, and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Months / Activity		2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1)	***Research***																		
	1) Bibliographic scientific research																		
	2) Market research																		
A2)	***Semi-finished sprayable base preparations***																		
	1) Microbial stability																		
	2) Rheological stability																		
	3) Shelf-life study																		
	4) Application on finished products																		
A3)	***Development of a completely new sprayable product***																		
	1) Pilot plant production																		
	2) Microbial stability																		
	3) Rheological stability																		
	4) Shelf-life study																		
	5) Technological characterization of the obtained final product																		
A4)	***Scaling up at an industrial level***																		
	1) Optimal Process																		
	2) Scale up																		
A5)	***Thesis and Paper Preparation***																		

3. Selected References

Lostie M, Peczalaski R, Andrieu J, Laurent M (2002). Study of sponge cake batter baking process. Part I: experimental data. *Journal of Food Engineering* 51: 131-137.

Martínez-Monzó J, García-Segovia P, Albors-Garrigos J (2013). Trend and innovations in bread, bakery, and pastry. *Journal of Culinary Science & Technology* 11: 56-65.

Mitelut A, Popa E E, Popescu P, Popa M E (2021). Trends of innovation in bread and bakery production. *Trends in Wheat and Bread Making* 7, 199-226.

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Exploitation of functional potential of autochthonous microorganisms from fermented foods

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This PhD thesis research aims to isolate new candidate probiotic strains possessing traits potentially useful to hinder NCDs, to introduce them in traditional and new functional foods.

Valorizzazione del potenziale funzionale di microrganismi autoctoni da alimenti a fermentazione naturale

Questo progetto di tesi di dottorato mira ad isolare nuovi ceppi probiotici in grado di ostacolare le malattie non trasmissibili, allo scopo di introdurli in alimenti funzionali tradizionali e non tradizionali.

1. State-of-the-Art

Nowadays consumer's demand for safe, high-quality, and health-promoting foods is growing more and more. Research has been demonstrating that nutrition can prevent several diseases, by reducing risk factors and increasing certain physiological functions. There are several studies that highlight the link between diet and a specific class of diseases, called non communicable diseases (NCDs). NCDs are slow-developing and long-lasting diseases, such as cancer, diabetes, cardiovascular, respiratory, and neuro-degenerative diseases, which are the cause of more than 71% of annual deaths worldwide. In recent years, scientific evidence that emphasizes the correlation between the development of NCDs and gut microbiota has emerged: microbiota dysbiosis is a common trait in people affected by these pathologies. Also, gut microbiota is closely related to nutrition. Among the microbiota species, it is particularly important to monitor the so-called next generation probiotics (NGPs): *Faecalibacterium pausnitzii*, *Akkermansia muciniphila* and *Eubacterium hallii* are the most promising. NGPs recently gain attention for their role in prevention and treatment of many chronic diseases. For example, *Akkermansia muciniphila* helps to prevent type 2 diabetes and to maintain glucose homeostasis (De Filippis et al., 2022).
From this perspective, functional foods can play a central role. Functional foods are defined as those foods which, besides providing nutrients and energy, are also able to enhance health or reduce the risk of disease. One of the main classes of functional foods are fermented foods. The main agents of fermentation are lactic acid bacteria (LAB), which play a key role in guaranteeing safety, enhancing shelf-life, and developing typical sensory properties of the product. Moreover, during fermentation, vitamins, minerals, biologically active peptides, bacteriocins, antioxidants are produced, and some anti-nutritional factors are removed. As a result, these products provide many health benefits. In traditional fermented foods fermentation occurs spontaneously, thanks to autochthonous microorganisms that are naturally present in the raw food and can be promising probiotic candidates (Grujović et al., 2021). There are several studies regarding the indigenous microflora, but studies focusing on their addition in functional foods with the aim to hinder NCDs are still lacking.
The potential of indigenous LAB from fermented foods is huge, but the selection of the best probiotic candidates to use in the development of a functional food is challenging. It is indeed necessary to consider functional, safety, technological and physiological aspects. As for the functional aspects, numerous studies have confirmed antioxidant, anti-inflammatory, immunomodulatory, anti-carcinogenic, anti-cholesterol, antidiabetic, and anti-obesity effects of natural microflora and their metabolites. LAB not only show several metabolic pathways useful for health, but they may also modulate gut microbiota by producing active metabolites (e.g., short chain fatty acids (SCFAs) and antimicrobial substances) in the gut, which contribute to prevent microbiota dysbiosis (Grujović et al., 2021). In this regard, in vitro models that simulate large intestine fermentations are crucial to assess the effect of probiotics on gut microbiota and its metabolites. As for safety aspects, probiotic candidates must obviously be safe for human consumption, so they must be non-pathogenic strains with no toxic properties. Most LAB are included in the list of species presumed to be safe, in accordance with the QPS (Qualified Presumption of Safety) concept introduced in 2007 by the European Food Safety Authority (EFSA). From a technological point of view, probiotics must be able to survive and maintain a high vitality in the food matrix during all processing steps and shelf-life, since the physiological effect is guaranteed only in the presence of high concentrations of viable cells (at least 10^6-10^7 CFU/g). Furthermore, in the case of fermented foods, they must not interfere with the metabolic activities of the indigenous microflora of the product, nor negatively influence the physico-chemical, structural, and sensory characteristics of the product. Lastly, to exert the health effects they are selected for, physiological

features are relevant. Probiotics must survive the gastrointestinal transit (GIT), which depends first on their tolerance to gastric acidity, antimicrobial activity of pepsin, bile salts and pancreatin (Marino et al., 2021).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this project can be divided into the following activities according to the Gantt diagram (Table 1):

A1) **Literature review** in order to select the most suitable food products for strains' isolation and a definitive list of functional activities to look for.

A2) **Isolation of autochthonous LAB strains from different food matrices and evaluation of their probiotic potential** in order to collect safe bacterial strains able to reach and interact with the target site. (A2.1) Safety assessment (absence of biogenic amines production, absence of horizontal transfer of antibiotic-resistance genes). (A2.2) Probiotic assessment: survival ability to GIT stressors (pH, gastric juices, and bile salts) and intestinal colonization ability (adherence to enterocytes, auto-aggregation, cell surface hydrophobicity, co-aggregation). (A2.3) Species assignment through 16S rDNA sequencing, and molecular fingerprinting.

A3) ***In vitro* evaluation of functional properties** (anti-cholesterol activity, ACE-inhibitory activity, immunomodulatory and anti-inflammatory activity, pro-Next Generation Probiotics activity, alfa-amylase inhibition, GABA production, folates production, EPS production, …) in order to collect the most promising bacterial cultures.

A4) **Development of new functional foods**. (A4.1) Selection of possible food targets (e.g., dairy products, plant-based products, …). (A4.2) Development of functional foods and assessment of the microbiological, physico-chemical, and sensory characteristics. (A4.3) Assessment of strains' viability during production process, shelf-life, and *in vitro* digestion. (A4.4) Assessment of the impact on gut microbiota and its metabolites.

A5) **Writing and editing of scientific papers and PhD thesis**.

Table 1 Gantt diagram for this PhD thesis project.

ACTIVITY/MONTHS		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
A1)	***Literature review***																																				
A2)	***Isolation of autochthonous LAB strains from different food matrices and evaluation of their probiotic potential***																																				
	1) Safety assessment																																				
	2) Probiotic assessment																																				
	3) Species assignment																																				
A3)	***In vitro* evaluation of functional properties**																																				
A4)	***Development of new functional foods***																																				
	1) Selection of possible food targets																																				
	2) Development of functional foods and assessment of the microbiological, physicochemical, and sensory characteristics																																				
	3) Assessment of strains' viability during production process, shelf-life, and *in vitro* digestion																																				
	4) Assessment of the impact on gut microbiota and its metabolites																																				
A5)	***Writing and editing of scientific papers and PhD thesis***																																				

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Seaweed derived phlorotannins to counteract plant microbial infections: synthesis, structure-activity-relationship and mode of action studies

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This PhD project is aimed at synthesizing seaweed derived phlorotannins in order to study their efficacy as antimicrobial agent for application in the field of plant protection. Efforts will be dedicated to understanding and verifying the mode of action of the synthesized molecules. The interaction of the compounds with known molecular targets in pathogenic microorganisms will be assessed through calorimetric analysis.

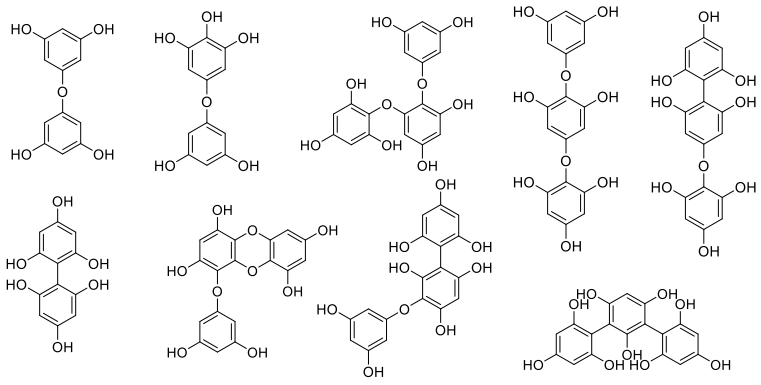
Florotannini derivati da alghe marine per contrastare le infezioni microbiche delle piante: sintesi, studi delle relazioni struttura-attività e della modalità d'azione

Questo progetto di dottorato è finalizzato alla sintesi di florotannini derivati da alghe marine al fine di studiarne l'efficacia come agenti antimicrobici per una potenziale applicazione come agrofarmaci naturali. Verranno inoltre effettuati studi volti alla comprensione e alla verifica della modalità d'azione delle molecole sintetizzate. Grazie all'analisi calorimetrica verrà valutata l'interazione dei composti con bersagli molecolari noti, presenti nei microrganismi patogeni.

1. State-of-the-Art

Plant diseases are caused by pathogenic microorganism, mainly fungi, which affect the quality and the yield of food produced. If we consider cereals, roots, tubers, pulses, fruits and vegetables the food losses can reach up to the 30% in all the stages of the crop production, as reported by FAO (Food and Agriculture Organization of the United Nations). Pathogen control has always been effectively performed mostly by synthetic fungicides. However, nowadays only a few of them are still allowed worldwide. As a consequence, the widespread use of a few classes of fungicides, due to the increase of agricultural production, has generated resistance in some pathogenic fungi (Nazarov *et al.*, 2020; Kunova *et al.*, 2016). Furthermore, synthetic pesticides have created a high environmental impact over the years, also causing bioaccumulation. Therefore, to address the growing urgency in managing fungal diseases, there is the need to find new compounds that can effectively fight pathogens and that have lower human and environmental impact as well. A promising approach could be the development of nature derived agrochemicals. In the last decades, phlorotannins, which are polyphenolic compounds present in brown seaweed, have emerged as a new class of antibacterial and antifungal natural compounds.
Phlorotannins are present in the seaweed extract as a very complex mixture of compounds, being them oligomers of phloroglucinol. This means that the phloroglucinol units are connected each other generating more complex structures such as diphenylethers, biphenyls and dibenzodioxines. Some representative structures are shown in Figure 1.

Figure 1 Structures of some low molecular weight phlorotannins.



Corato *et al.* demonstrated that crude brown algae extracts have higher inhibitory activity against *B. cinerea*, *M. laxa* and *P. digitatum* than red algae extracts, reaching 100% inhibition at concentration of 30 g/L of exctract (De Corato *et al.*, 2017). However, the antifungal potential of the extract has not been conclusively attributed to specific

constituents, and data on the mode of action are still missing. Interestingly, from a survey of the literature it emerged that dieckol, a phlorotannin extracted from the brown seaweed *E. cava* showed antifungal activity against human fungi, with a MIC of 200 μM (Lee *et al.*, 2010).The authors also suggested that the antifungal activity was due to the changes in the integrity of the cell membrane, causing its disruption. The activity of brown seaweed extract on plant pathogenic fungi is supposed to be related to the presence of phlorotannins as well, though there is no clear evidence of the antifungal activity of pure compounds, since they are present in low amount in the algal matrices and their isolation is quite troublesome. Surprisingly, the synthesis of most of the molecules belonging to this class of compounds is not reported in the literature. Thus, this PhD project will be focused on the development of efficient synthetic strategies to obtain selected phlorotannins in sufficient amount to perform biological tests. Furthermore, the interaction of the synthesized molecules with a model membrane, representative of plant pathogenic fungi membrane, will be evaluated through calorimetric analysis to elucidate the mechanism of action of phlorotannins. To the best of my knowledge, this approach, associated to the thermodynamic analysis of the interaction, has never been applied yet to evaluate the action of compounds with model fungal membranes. Upon activity evaluation, the most promising compounds will be selected for chemical functionalization to improve their antifungal profile. Indeed, following the thermodynamic information (Saitta *et al.*, 2021), a series of chemical modifications could be performed to the synthesized phlorotannins as to achieve satisfactory activity. Furthermore, if our thermodynamic studies evidenced that the membrane is not the target of some of our compounds, other potential targets would be considered.

2. PhD Thesis Objectives and Milestones

Within the general aims mentioned above, the research activity can be divided into the following chronologically overlapping and interdisciplinary activities, according to the Gantt diagram showed in Table 1:

A1) **Total synthesis of natural phlorotannins**, which also includes optimization of the overall synthetic pathway and structure elucidation through Nuclear Magnetic Resonance (NMR) and Mass Spectrometry techniques.

A2) **Biological evaluation of antifungal activity** will be performed in collaboration with plant pathologists and will assess the ability of synthesized compounds to affect mycelia growth and spore germination inhibition.

A3) **Evaluation of the interaction of pure phlorotannins with phospholipid model membranes**, in order to establish the mechanism of action of selected natural compounds. The effect of different phlorotannins, as pure compounds, will be evaluated, by Differential Scanning Calorimetry, on liposomes representative of fungi's real membranes (A3.1) as well as on simpler vesicles in order to dissect the mechanism of interaction (A3.2).

A4) **Structural optimization process** based on chemical modification of natural phlorotannins will be performed in order to increase their activity. The obtained derivatives will be tested again to evaluate their efficacy.

A5) **Writing and Editing of the PhD thesis**, scientific papers, oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Phlorotannins Synthesis***																								
A2)	***Antifungal activity evaluation***																								
A3)	***Interaction evaluation***																								
	1) Representative vesicles																								
	2) simpler vesicles																								
A4)	***Structure optimization***																								
A5)	***Thesis and Paper Preparation***																								

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Anti-Quorum Sensing Activity of Probiotics against Foodborne-Spoilage and Pathogenic Bacteria

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Quorum sensing (QS) is a mechanism for cell-to-cell communication between inter- and intra-bacterial species that is employed to regulate the gene expression of virulence factors. Microbial control through QS inhibition is a rising interest of research. However, utilisation of probiotics as a source of QS inhibitors is under-researched, in particular, against food spoilage bacteria. This project aims at the following: 1) to identify the type of QS in isolates of foodborne-Pseudomonas and Campylobacter species. 2) to investigate the anti-QS activity of probiotics against these isolates. 3) to enhance the anti-QS activity through microencapsulation.

Attività di rilevamento anti-quorum sensing dei probiotici contro i batteri patogeni e il deterioramento di origine alimentare

Questo progetto di tesi di dottorato mira a mettere a punto un procedimento sperimentale, in batch o a riciclo totale, prima in impianto da banco e poi in impianto pilota, atto ad individuare il modello matematico in grado di simulare il processo di recupero di selezionati biopolimeri di interesse alimentare mediante moduli a membrana di ultrafiltrazione tubolari o a fibre cave, consentendone il dimensionamento ottimale in scala industriale.

1. State-of-the-Art

Quorum sensing is a cell-to-cell communication that occurs between intra-and inter-bacterial species and is regulated by signalling molecules called autoinducers (AIs). When the AIs reach a certain threshold of levels, they bind to protein receptors, which then initiates QS circuit. There are three common types of autoinducers, which are produced for initiation of QS. The first is acyl-homoserine lactones (AHLs) that is produced by *luxI* gene in Gram-negative bacteria. The second is autoinducer peptides (AIP) that is produced by *agrD* gene in Gram-positive bacteria. The third one is called AI-2 that is synthesized by *luxS* gene, and this can be present in Gram-negative and Gram-positive bacteria.

Bacterial communication in foods through QS plays a role in the food quality and safety. That is, production of virulence factors by foodborne-pathogenic and spoilage bacteria is regulated by QS mechanism. For example, spoilage of dairy products by biofilm and enzymatic activity (proteolytic, and lipolytic) of *Pseudomonas* species (*P. fluoroscens, P. fragi, P. gassari, P. putida* and *P. lactis*) is regulated by AHL-type QS system (Quintieri et al., 2021. In addition, spoilage in meat and vegetables is also induced by Gram-negatives, where acyl-homoserine lactones (AHL) and AI-2 act as signalling molecules for QS induction. The AHL autoinducers (C_4-HSL, C_6-HSL and C_6-3-oxo-HSL) were detected in chilled-stored meat products and vegetables (broccoli, parsley, carrots, and spinach) spoiled by *Enterobacteriaceae* and *Aeromonas* species. In addition, AI-2 activity was also found in meat products contaminated with *Pseudomonas fragi* and *E. coli* O157:H7 (Skandamis *et al*., 2012). Milk can be also contaminated with pathogenic Gram-positive bacteria such as *Listeria. monocytogenes* that uses peptides (i.e., AIP) as QS signalling molecules to trigger gene expression for biofilm formation (Bai *et al*., 2021).

With the widespread use of antibiotics in animal industry, spread of antimicrobial-resistance bacteria in foods is of a major concern for food industry. Microbial control through QS inhibition is an alternative strategy to overcome antimicrobial resistance of bacteria due to the fact that QS disruption would inhibit expression of virulence-related genes without growth inhibition. A wide range of plant-derived compounds (such as phenol acids) and QS signal-degrading enzymes were demonstrated as QS inhibitors (QSIs) (Machado *et al*., 2020). Oregano essential oil, cinnamaldehyde and catechins inhibited the expression of virulence factors by *P. fluorescens* (extracellular protease activity, biofilm formation, swarming and swimming motility) through anti-QS activity (Rossi *et al*., 2018; Ding *et al*., 2019). In addition, in-vitro studies showed that probiotics are a promising source of QS inhibitors against foodborne pathogens (Davares *et al*., 2022). However, in-situ studies investigating anti-QS activity of probiotics is yet to be demonstrated. Furthermore, QS inhibition in foodborne-spoilage bacteria is still lacking. Indeed, QS inhibitors from probiotics can be utilised as a sustainable and safe method of food preservation.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) **Isolation of foodborne pathogenic and spoilage bacterial strains from dairy and meat products** (*Pseudomonas* and *Campylobacter* species), *Pseudomonas* species are main spoilage species in meat and dairy products. *Campylobacter* species are causative of Campylobacteriosis, which is the main foodborne illness in the European union in the last few years. Assessment of virulence factors of the isolates such as enzymatic activity and biofilm formation.

A2) **Identification of the type of QS system** in the isolated *Pseudomonas* and *Campylobacter* strains

A3) **Investigation of the anti-virulence and anti-QS activity** of a wide range of probiotics *lactobacillus* and *Bifidobacterium* strains (as planktonic cells, or their metabolites and cell-free extract) against the isolates to select the most effective probiotic strains.

A4) **Transcriptomic analysis** of QS-related and virulence-related genes to study the change in genes expression after treatment with probiotics (in-vitro and in situ).

A5) **Studying the effect** of microencapsulation on the anti-QS activity of probiotics strains and the expression of genes encoding production of QS inhibitors.

A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Isolation of Target Bacteria***																								
	1) Isolation of *Pseudomonas* and *Campylobacter* species																								
	2) Assessment of virulence factors																								
A2+3)	***Screening of QS Systems in the Isolates***																								
	1) Identification of the type of QS system using bioreporter strains																								
	2) Investigation of anti-QS activity of probiotics																								
A4)	***Transcriptomic Analysis***																								
	Transcriptomic analysis of QS-related genes																								
	Transcriptomic analysis of virulence-related genes																								
A4)	***Effect of Microencapsulation on QS***																								
	1) Effect of microencapsulation of QS-related genes expression in probiotics																								
	2) Anti-QS activity of probiotics microcapsules																								
A5)	***Thesis and Paper Preparation***																								

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Development of organogels containing bioactive compounds from agri-food by-products and their application for innovative and sustainable foods formulation

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This PhD thesis aims at developing organogels containing bioactive compounds from agri-food by-products, in order to obtain an innovative and sustainable solution for the replacement of traditional solid fats currently employed in food formulations. This project can help valorizing agri-food by-products and developing innovative food products that are safe, stable (from a physico-chemical and oxidative standpoint), with at least the same, or even better, shelf-life, organoleptic and nutritional characteristics of the corresponding conventional products.

Sviluppo di organogel contenenti composti bioattivi da sottoprodotti e loro applicazione per la formulazione di alimenti innovativi e sostenibili

Il presente progetto di ricerca si propone di sviluppare organogel contenenti composti bioattivi derivanti da sottoprodotti della filiera agro-alimentare, che forniscano una soluzione innovativa e sostenibile per la sostituzione di grassi solidi tradizionali attualmente impiegati nelle formulazioni alimentari, permettendo così di valorizzare i suddetti sottoprodotti e di sviluppare alimenti innovativi che siano sicuri, stabili da un punto di vista chimico-fisico ed ossidativo, e che abbiano almeno le stesse, se non superiori, caratteristiche di conservabilità, organolettiche e nutrizionali dei prodotti convenzionali.

1. State-of-the-Art

In the past few years, legislative limitations to the use of fats rich in *trans* fatty acids (TFA) in food, the rising awareness of consumers regarding the negative effects of TFA and saturated fatty acids (SFA) on human health and the environmental impact related to the large use of palm oil in food and biodiesel productions, oriented researchers to study alternative lipid-based structures that were healthier and had a lower environmental impact (Li *et al*., 2022). However, SFA and TFA play an important role in foods, as they confer different properties such as plasticity, taste, flavor, mouthfeel, texture, etc. Thus, there is a real need to find solutions for the replacement of the so-called "hard" fats (generally with a high content of SFA and/or TFA) in foods, but without compromising technological and sensory characteristics of the later. One of the main alternatives that have been proposed are organogels, which are gels in which a continuous liquid phase (vegetable oil or water) is entrapped and immobilized in a thermo-reversible three-dimensional network through the use of non-triglyceridic organogelators (Bascuas *et al.*, 2020). Organogels have been used as replacers of traditional solid fats in several food products (baked products, meat products, dairy products etc.) to reduce the total amount of fat (particularly of SFA and TFA), still giving a solid texture. In fact, the structuring mechanism of organogels does not change the chemical composition of the starting liquid phase nor its nutritional value, which is one of the main advantages with respect to other widely used fat structuring processes in food industry, such as hydrogenation and interesterification (Li *et al*., 2022). Moreover, many studies have positively evaluated their utilization as "carrier systems" for the transport and retention of bioactive compounds. Indeed, organogels may represent a system potentially capable to increase their solubility and dispersibility within food matrix and their bioavailability in the gastro-intestinal tract, by controlling their release and protecting them from oxidation and loss of functionality (Orhan and Eroglu, 2022). In this context, the agri-food by-products, to which are attributed high disposal costs and a low market value for their reuse, represent a significant source of bioactive compounds with high biological value; in fact, thanks to their proven antioxidant, antimicrobial and health properties, they could be used as ingredients and/or additives in food formulations (Fritsch *et al*., 2017). Therefore, adhering to circular economy, green economy and sustainability concepts, organogels represent an interesting alternative for the inclusion and subsequent valorization of agri-food by-products. The main by-products at both Italian and European levels are those from grain, olive oil, tomato and potatoes (Fritsch *et al*., 2017), which are rich in bioactive compounds such as carotenoids, phenolic compounds, etc. Lastly, organogels may be used in the formulation of plant-based food, an emerging market trend that is perceived by the consumers as more sustainable food solutions and more adherent to ethical, environmental sustainability, and health-nutritional aspects (McClements and Grossmann, 2021). These products could represent an interesting application for bioactive compounds-loaded organogels, especially if we

consider that they have to deal with a "flavor challenge" as they are often characterized by a high presence of off-flavors that can be generated by lipid and/or protein oxidation during processing (Leonard *et al*., 2022; Wehrmaker *et al*., 2022). In the formulation of innovative foods (conventional and/or plant-based), the use of organogels rich in bioactive compounds from agri-food by-products may represent an application capable of improving the shelf-life, slowing and/or drastically reducing oxidative and hydrolytic processes of lipids and proteins, with positive impact on organoleptic quality and health-nutritional characteristics of these food products, while valorizing agri-food by-products and enhancing the sustainability of the supply chain.

2. PhD Thesis Objectives and Milestones

The goal of this PhD thesis research is to develop organogels containing bioactive compounds from agri-food by-products, in order to find an innovative and sustainable solution for the replacement of traditional solid fats currently used in food formulations. This project may enable the valorization of agri-food by-products and the development of innovative and safe food products, that have to meet the same, if not superior, standards for stability (from a physico-chemical and oxidative point of view), shelf-life, organoleptic and nutritional characteristics as conventional products.

The PhD thesis project can be divided into the following activities, summarized in the Gantt diagram shown in Table 1:

A1) Bibliografic research
A2) Extraction and characterization of bioactive compounds from agri-food by-products
A3) Development of organogels including bioactive compounds
A4) Formulation of innovative foods (conventional and/or plant based)
A5) Shelf-life study of selected products
A6) Writing and publication of the PhD thesis, posters, scientific papers and oral presentations

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Bibliografic research***																								
A2)	***Extraction and characterization of bioactive compounds from agri-food by-products***																								
A3)	***Development of organogels including bioactive compounds***																								
	1) Evaluation of physico-chemical and oxidative stability																								
	2) Evaluation of organogels' retention efficiency of selected bioactive compounds																								
A4)	***Formulation of innovative foods***																								
	1) Setting up of products' formulation/s with pre-selected organogel/s																								
	2) Evaluation of the composition, stability (physico-chemical and oxidative) and sensory profile on innovative products																								
A5)	***Shelf-life study of selected products***																								
A6)	***Thesis and Paper Preparation***																								

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Novel Algorithms and Software Tools for LR-NMR Applications in Food Science and Technology

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This PhD research project is aimed at the development of software tools for the evaluation of Low Resolution (LR) Nuclear Magnetic Resonance (NMR) data pertinent to applications in food science and technology. This software would be a necessary tool to develop new applications and for the optimisation of the analysis in terms of precision and reproducibility of the results. The software, written in C++, will include basics operations along with innovative algorithms, which will be tested on both simulated and experimental data.

Nuovi Algoritmi e Strumenti Software per Applicazioni NMR a Bassa Risoluzione in Scienze e Tecnologie Alimentari

Questo progetto di ricerca di dottorato prevede lo sviluppo di strumenti software per l'elaborazione di dati di Risonanza Magnetica Nucleare a Bassa Risoluzione pertinenti ad applicazioni in scienze e tecnologie alimentari. Il software sarebbe uno strumento necessario per lo sviluppo di nuove applicazioni e per l'ottimizzazione del processo di analisi in termini di precisione dei risultati e della loro riproducibilità. Il software, scritto in C++, comprenderà operazioni di base a fianco di algoritmi innovativi, i quali verranno testati con dati sia simulati che sperimentali.

1. State-of-the-Art

Food technology impacts on all steps of food processing, starting from the production of foodstuffs, to their storage, various transformations, and even cooking. Each step must include proper concurrent quality assessment and safety controls. Nuclear Magnetic Resonance (NMR) is a very useful method to study and characterize several chemical and physical properties of the soft matter, including all kinds of materials and therefore also foodstuffs. The salient features of NMR include a large penetration depth, a totally non-invasive nature, the capability to discriminate even small variations in chemical composition as well as in molecular aggregation and mobility, an intrinsic quantitative response and good reproducibility. The drawbacks of NMR, in some contexts, are its relatively low sensitivity and the need to apply a relatively strong and very homogeneous magnetic field.
NMR comprises three distinct branches: relaxometry, spectroscopy, and imaging. Relaxometry studies the temporal evolution of nuclear magnetization and the ways it is affected by the molecular dynamics of the sample, spectroscopy is concerned mostly with highly resolved radio spectrum of a sample which reflects its chemical properties (molecular structure and composition), and imaging specializes in obtaining various kinds of visual images of the internal parts of a sample.
NMR have been widely used to solve many problems in the general area of food technology. While in NMR spectroscopy many useful high-resolution applications were developed by focusing on the chemical assignments and quantification of various spectral peaks, in NMR relaxometry the situation is different. There exist hundreds of publications proposing various Low Resolution (LR)-NMR applications related to food quality and processing, but relatively few of these potential applications were so far actually refined to the stage of practical assessment procedures.
LR-NMR applications cover many recognizable categories, such as the distinction of sample components or phases (muscle/fat, solid/liquid) or inner states (ripe or damaged), rheological and textural properties (Glicerina *et al.*, 2017) monitoring melting/freezing processes or diffusion processes (Bertram *et al.*, 2005), determination of particle/droplet sizes in emulsions (milk, cream), ageing of materials (stocked food, cheese ripening), or even the assessment of products authenticity, as Mengucci *et al*. (2021) carried out on PDO buffalo mozzarella cheese.
Despite the great number of applications, there are not many complete software tools dedicated to data analysis for specific use in industries. LR-NMR instruments are usually equipped with different hardware features from each other and with low software support to any particular application. In general, data format and the evaluation procedures do not follow any universal standard. In this situation application developers struggle and find difficult to guarantee reproducibility of the results. So, there is a great need for a uniform, vendor-agnostic software tool, one sufficiently sophisticated to allow an expert user, once he selects a potential application, to optimize it, to assess its precision and its reproducibility, to automate it, and to make it suitable for practical use in industrial environments.
In this PhD I plan to develop a complete package for the optimization of some specific LR-NMR applications in food science and technology. I will select some specific food or products; I will do sampling and acquisition of

data. Then I will develop software dedicated to the data analysis, in order to obtain results which ought to be reliable and reproducible. The entire process, from data acquisition to results, will be proposed as a complete method for a practical use in an industrial environment.

2. PhD Thesis Objectives and Milestones

An application developer employs one or more LR-NMR instruments to acquire data and a software tool for the data analysis, in order to optimize and automate the whole process. The goal of this project is the development of high-level software tools for the evaluation of LR-NMR data pertinent to possible applications in food technology and testing. This would provide support for the development and optimisation of specific applications. The software will be mostly written in C++ and will be based on either innovative algorithms and on improvements of the existing ones, and it will be focused on obtaining quantitative information about the sample physical and chemical properties, structure, quality. Simulated data will be generated to test the algorithms correct operation; then, real data will be acquired to test the effective robustness and stability of the software routines. The quality of the results achieved during the PhD course will be verified through the feedback received from potential users, who will employ the alpha version of the application software to analyse food products selected for verification.

The doctoral project may be organised in the following activities, resumed in the Gantt chart shown in table 1:

A1) Preparation: bibliographic research about LR-NMR applications in food science and technology.
A2) Software development: research of the currently available software tools for NMR data evaluation, research of innovative algorithms and their implementation and testing.
A3) Experiments: data acquisition and sequence optimization.
A4) Application development: choice of one or two potential applications to optimise in terms of analysis workflow from the data acquisition to the final extrapolation of results.
A5) Writing and publishing: scientific papers, posters, final thesis and oral presentation.

Table 1 *Gantt diagram for this PhD thesis project*.

Activities	Month	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1)	***Preparation***																		
	1) Bibliographic research																		
A2)	***Software development***																		
	1) Research of currently available software tools and algorithms																		
	2) Software project and implementation																		
	3) Testing algorithms																		
A3)	***Experiments***																		
	1) Data acquisition																		
A4)	***Applications development***																		
A5)	***Preparation of papers and thesis***																		

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Generating Novel Synbiotic Formulations with Multi-Functional Features: A Focus on Psychobiotic Effects

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The proposed PhD project aims to develop new synbiotic formulations combining probiotics and prebiotics to enhance gut health, immune function, and mental well-being. In vitro analyses will be conducted to evaluate the safety, stability, and efficacy of these formulations using experimental models that simulate gut-brain axis. Promising formulations will then undergo in vivo studies in animal models to assess their impact on mental health and cognitive function. This research has the potential to contribute to the creation of multi-functional synbiotics that can improve human mental health and cognitive performance, leading to development of new dietary supplements and functional foods.

Generazione di nuove formulazioni simbiotiche con caratteristiche multifunzionali: Un focus sugli effetti psicobiotici

Il progetto di dottorato proposto mira a sviluppare nuove formulazioni simbiotiche che combinino probiotici e prebiotici per migliorare la salute dell'intestino, la funzione immunitaria e il benessere mentale. Saranno condotte analisi in vitro per valutare la sicurezza, la stabilità e l'efficacia di queste formulazioni utilizzando modelli sperimentali che simulano l'asse intestino-cervello. Le formulazioni promettenti saranno poi sottoposte a studi in vivo su modelli animali per valutare il loro impatto sulla salute mentale e sulla funzione cognitiva. Questa ricerca ha il potenziale per contribuire alla creazione di sinbiotici multifunzionali in grado di migliorare la salute mentale umana e le prestazioni cognitive, portando allo sviluppo di nuovi integratori alimentari e alimenti funzionali.

1. State-of-the-Art

The human body is home to a diverse microbial ecology hosting about 90% microbial cells and 10 million microbial genes. The gastrointestinal tract, the most colonized portion of the human body, is the site of a complex and mutualistic relationship between the microbial habitat and the host, resulting in a health-promoting stable community (Kelly et al., 2016). Recent research has focused on the manipulation of the gut microbiota using various interventions, including probiotics, prebiotics, and their combination (synbiotics), to enhance health and treat various disorders (Allen et al., 2017). Synbiotics work synergistically to improve the microbial eubiosis, showing promising results in various disorders, including mental health disorders, such as depression and anxiety (Aggarwal et al., 2013).

Nowadays, research interest is gradually shifting to probiotics able to improve mental health thanks to their positive effect on the gut-brain axis. In the specific, several studies have shown that certain bacteria, including lactobacilli and bifidobacteria, can improve depressive symptoms in animal models and human clinical trials (Kelly et al., 2015). This subcategory of probiotics, defined as psychobiotics, confers a mental health benefit to the host showing a direct effect on brain function and behavior. Psychobiotics work through various mechanisms of regulation of the gut-brain axis, including regulating the production of neurotransmitters, reducing inflammation, and modulating the hypothalamic-pituitary-adrenal axis, which is a key regulator of the stress response and mood regulation (Dinan et al., 2013).

Moreover, recent research has focused on the development of novel synbiotic formulations with multi-functional features, with an emphasis on their psychobiotic effects. These formulations have shown promising results in various disorders, including mental health disorders and metabolic disorders (Schmidt et al., 2015). However, more studies are needed to optimize the formulation and dose of these synbiotics, as well as to discover the precise bacterial strains and prebiotic fibers that are most effective for their therapeutic effects and to develop individualized treatments for maximum efficacy.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Literature review and microbial strains selection**
Understanding the laboratory organization; reviewing the latest scientific literature related to my project and defining of the main research topics and selection of bacteria and yeasts strains from Micro4Food culture collection.

A2) **Screening of the strains' performance and phenotypic characterization**
Screening of strains will be modulated by evaluating the performance of each strain through different activities like evaluating the gastrointestinal transit resistance, the enzymatic activities, the growth kinetics, and the acidification rates. The molecular characterization of glutamic acid decarboxylase (GAD) gene will be done as pre-selection for gamma-aminobutyric acid (GABA) producing strains. Microbial strains will also be characterized phenotypically to understand their carbon and nitrogen consumption profiles by using OmniLog Phenotype MicroArray (PM) Technology.

A3) ***In vitro* analyses**
Once the screening of the strains will be completed, the most promising probiotic strains will be selected using a statistical approach. Afterwards, the potential psychobiotic effect of the strains will be evaluated through the characterization of neurotransmitters and short chain fatty acids (SCFA) produced through fermentation Moreover, the anti-inflammatory response and the effect on the intestinal barrier permeability from the intervention using the candidate probiotic strains through in vitro analyses will be evaluated.

A4) **Hypothalamic Pituitary Adrenal (HPA) axis and serotonin production**
Evaluating the HPA axis in response to psychobiotics will help to determine if the microbial strains can affect stress hormone levels, regulate the stress response, and potentially alleviate stress-related conditions. To investigate this, adrenocorticotropic hormone (ACTH) stimulation test and gene expression analysis will be performed *in vivo.* The production of serotonin, a key neurotransmitter involved in mood regulation and mental well-being, will also be examined by using germ free mice.

A5) **Writing and Editing**
Participating to national and international scientific conference for oral and/or poster communications; writing and editing of the PhD thesis and scientific papers.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Literature review and strains selection***																								
A2)	***Screening of the strains***																								
	1) Strains performance																								
	2) Phenotypical characterization																								
A3)	***In vitro analyses***																								
A4)	***HPA axis and serotonin production***																								
A5)	***Papers publication and thesis writing***																								

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A "green path" strategy for complete recovery of buckwheat processing byproducts

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The aim of this Ph.D. project is to set up a sustainable recovery process to valorize as much as possible residuals from buckwheat processing. Buckwheat husk has a high content of polyphenolic compounds and dietary fibres. We propose a sequential approach. A first step is the extraction of compounds to be used as functional ingredients in the formulation of novel foods. A second step plans to recover the cellulosic fraction from the residual lignocellulosic material, and to evaluate its applications in various fields due to its mechanical properties, reinforcing capacity, and biodegradability.

Un approccio "green" per la valorizzazione dei sottoprodotti: una proposta per il recupero completo dei sottoprodotti della lavorazione del grano saraceno

Il presente progetto di dottorato mira a valorizzare lo scarto residuo derivante dalla lavorazione del grano saraceno attraverso processi che hanno un basso impatto ambientale. La pula di grano saraceno presenta un alto contenuto di composti polifenolici e fibre alimentari. Proponiamo un approccio sequenziale che prevede come primo step l'estrazione di composti che possono essere utilizzati nella composizione di nuovi alimenti come ingredienti funzionali. La frazione lignocellulosica residua sarà utilizzata tal quale o per il recupero della frazione cellulosica che trova applicazioni in diversi campi, grazie alle sue proprietà meccaniche, capacità di rinforzo e biodegradabilità.

1. State-of-the-Art

Buckwheat is a summer-growing pseudocereal belonging to the Polygonaceae family, that complete its life cycle in less than 3/4 months, it has a good climate adaptability, with a preference for cool summers. Among the many buckwheat species, only two are cultivated for human consumption: common buckwheat (Fagopyrum esculentum) and tartary buckwheat (Fagopyrum tataricum), the latter characterized by a bitter taste and smaller seed (Siracusa et al. 2017). Buckwheat is an healty gluten-free grain, so it is suitable for the diet of people suffering from celiac disease or other gluten related disorders. Buckwheat has a high nutritional value and health beneficial properties, is a source of edible fibres, minerals, low-digestibility carbohydrates, proteins with high biological value, vitamins such as B1, B2 and B6 and essential amino acids (Mota et al. 2017; Dziedzic et al. 2018). The other bioactive ingredients of this pseudocereal include phytosterols, squalene, phagopyritols, polyphenols and flavonoids. Unlike other cereals in which the phenols are mainly attached to cell wall components, the flavonoids in tartary buckwheat, including rutin, quercetin and quercitrin, are commonly present in the free form (Siracusa et al. 2017). Food and agro-industrial buckwheat processing results in a number of byproducts. Husk is a high-volume byproduct of decortication - the first step of buckwheat processing - and is a valuable source of bioactives, including polyphenols and dietary fibers. Husk has a much higher antioxidant activity than dehulled seeds, making its addition to novel formulations beneficial to humans (Dziadek et al. 2016) and to the food industry. However, implementing such a strategy implies production of bioactive-enriched materials without the intrinsic limitations of the original husk. Studies on the industrial-scale recovery of bioactive fractions in this context are relatively scarce, and the application of "green" procedures has been explored on a very sporadic and semi-empirical basis.

2. PhD Thesis Objectives and Milestones

The work will involve six main activities, as listed in what follows.

A1) **Literature research**: research, reading and comprehension of the most recent publications related to the project.

A2) **Macromolecular and micromolecular characterization of byproducts**. The hulk biomass will be ground and sifted for assessing the total dietary fibers content (A2.1). The same materials will be used for extraction of phenolics in acidified water, concomitantly with either ultrasonic treatment (Ultrasonic Aassisted Extraction, UAE) or Microwave Assisted Extraction (MAE) (A2.2).

A3) **Polyphenolic fraction characterization.** Total phenolics in the different samples will be assessed by the Folin-Ciocalteau assay. The total antioxydant activity will be measured through two different radical

scavenging method, DPPH and ABTS. The phenolics profile of the extracts will be determined by HPLC (A3.1). Subsequently, the potential protective effect of the extracts on cell inflammation will be evaluated on an established Caco-2 cell model, along with the inhibitory capacity against enzymes responsible for glucose metabolism. (Abbasi-Parizad et al. 2020, Capraro et al. 2021) (A3.2). The possible use of the solid residual from phenolics extraction as an ingredient of fiber-enriched gluten-free extruded product will be tested on blends with maize or rice, and products will be evaluated in terms of consistency, water retention capacity, viscosity, flavour quality and sensory traits (A3.3).

A4) **Recovery of specific macromolecules by mechanical and biotechnological treatments**. Mechanical and/or biotechnological treatments will be used to recover other valuable compounds, with a specific focus on cellulose fibrils (A4.1). Physical properties of the cellulose fibers will be assessed to provide guidelines for their use as ingredient in packaging and non-packaging products (A4.2).

A5) **Definition of prototype end products and of their potential**. Development of prototype product from isolated cellulose fibril and solid residual fraction, for obtaining environmentally friendly and biocompatible products in packaging and non-packaging field.

A6) **Data dissemination** of the obtained data in scientific papers and conference. PhD thesis writing.

Table 1 Gantt diagram for this PhD thesis project.

	ACTIVITY	DURATION (months)
A1)	*Literature research*	
A2)	*Macromolecules and small compounds characterization of byproducts*	
	1) Dietary fibers determination	
	2) Application of green technologies for polyphenol extraction	
A3)	*Polyphenolic fraction characterization*	
	1) Evaluation of antioxydant properties and profiling	
	2) Evaluation of anti-inflammatory properties	
	3) Novel uses in food and non-food product	
A4)	*Recovery of specific macromolecules by mechanical and biotechnological treatments*	
	1) Mechanical and/or biotechnological treatment	
	2) Characterization of the extracted cellulose fraction and residual material	
A5)	*Definition of prototype end products*	
	1) End use of isolated cellulose fibrils	
	2) Packaging end use of residual fraction	
A6)	*Data dissemination and PhD thesis writing*	

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36

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Human diet and digestion: a stoichiometry approach and gut microbial system dynamics

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The objective of this PhD project is to develop a new approach to describe the diet and the digestive process by a process-based model. A stoichiometric description and 13 C NMR spectroscopy will be employed to characterize the diet. Additionally, a mathematical model will be developed describing the interactions between the diet and the intestinal microbial community dynamics.

Dieta e digestione: approccio stechiometrico e dinamica della comunità microbica intestinale

L'obiettivo di questo progetto di dottorato è sviluppare un nuovo approccio di studio per la descrizione della dieta e del processo digestivo mediante un modello process-based. Una descrizione stechiometrica e la spettroscopia NMR a stato solido verranno utilizzate per caratterizzare la dieta. Parallelamente si svilupperà un modello matematico che descriva le interazioni tra la dieta e le dinamiche della comunità microbica intestinale.

1. State-of-the-Art

1.1 Stoichiometry and 13 C NMR approaches to describe human diet

Dietary intake is widely identified as one of the most important lifestyle factors influencing human health at planetary scale. So far, human diet studies have been focused on macronutrients and calories composition (Dai et al, 2022; Diaz-Ruiz et al, 2021), with a specific emphasis on health (Katsouyanni et al, 1994; Studnicki et al, 2019). Likewise, (Schmidhuber et al, 2018) realized a comprehensive database of the macro and micronutrient distributions at country level, giving an overview of the global trends over time. Chemical stoichiometry and NMR spectroscopy have received considerable attention in ecology, environmental sciences, forestry, evolutionary biology and plant soil sciences; however those approaches have been less explored in describing human diet composition.

1.2 Modelling gut diversity and functioning

As all ecological consumers are limited by the quality of available resources, ample evidence shows that the human microbiome composition reflects human diet quality. As complex adaptive systems, microbial communities show higher-order properties that are not present in individual microbes, arising from their interactions (Song et al, 2014). In this context, predictive mathematical models are helpful to understand the underlying principles of the dynamics and emergent properties of microbial systems. In table 1, the main modelling approaches applied to gut microbial ecosystems are reported.

A main group of models is constituted by generalized Lotka Volterra models (gLV), describing the growth of the individuals, expressed as taxa or operational taxonomic unit (OUT), in the gut community and their interactions (Stein et al, 2013); (Chung et al, 2017). Those kind of models have been widely applied, with differences in the measure units utilized (Joseph et al, 2020), with the addition of terms representing an immigration effect (Li et al, 2021), and also considering the interference of diet and antibiotics (Joseph et al., 2020). Otherwise, some researches and tools (Moorthy & Eberl, 2017) have been developed about gut microbial community dynamics focusing on their dependences on resources availability in terms of macronutrients (Kettle et al, 2015; Moorthy & Eberl, 2017). Along with these methods, individual based models (IBMs), widely used in ecology for modelling both higher level organisms and, more recently, microbes (Kang et al, 2014), were also applied to the gut ecosystem (Shashkova et al, 2016). A new definition of the diet pattern allows a different insight in the prediction and description of the interactions within the gut microbiota. In this context, this PhD project aims to propose a new mathematical model of the gut microbial dynamics and its relationship with the input resources described by the novel application of stoichiometry and 13 C NMR spectroscopy.

Table 1 Main mathematical models applied to the gut microbial communities dynamics

Modelling approach	Interaction units	State variables	Measure units
ODE	Individuals	Taxa	Relative concentration
ODE	Individuals	OTU	Absolute concentration
ODE	Individuals and resources	Bacterial functional groups and Polysaccharides	Absolute concentration
IBM	Individuals and resources	Species and Polysaccharides	Absolute concentration

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) **New approaches to describe human diet pattern** using the stoichiometric and 13 C NMR spectroscopy methods. Stoichiometry of Carbon and Nitrogen is applied to create a new global database of the human diet (A1.1) and then used to define different types of diets (A1.2). Then, to improve the carbon quality characterization of the human dietary pattern 13 C NMR spectroscopy can be used (A1.3).

A2) **Identification of microbial community in the gut** using a process-based modelling approach. The model will be defined as the interaction between the microbiome species and the food intake expressed as macronutrients. The process of modelling will include the step of data analysis (A2.1), model design and calibration (A2.2), and model calibration and validation (A2.3).

A3) **Identification of microbial community and food dynamics in the gut** using a process-based modelling approach**.** In this phase the model will be defined as the interaction of the microbiome community and the food intake dynamics including its digestion in the gut. The process of modelling will include the step of data analysis (A3.1), model design and calibration (A3.2), and model calibration and validation (A3.3).

A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***New approaches to describe human diet pattern***																								
	1) Create a global database of C/N ratio in human diet																								
	2) Calculate the C/N ratio associated to different diet																								
	3) NMR spectroscopy of human diet																								
A2)	***Microbial community dynamics in the gut***																								
	1) Data analysis																								
	2) Model design and implementation																								
	3) Model calibration and validation																								
A3)	***Microbial community and food dynamics in the gut***																								
	1) Data analysis																								
	2) Model design and implementation																								
	3)Model calibration and validation																								
A4)	***Thesis and Paper preparation***																								

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Phenotypic and genotypic diversity among potential next-generation probiotics

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The modulation of the gut microbiota is emerging as a promising target for the management or prevention of many diseases, especially by using traditional probiotics such as Lactobacilli and Bifidobacteria. This PhD thesis research project aims to isolate and investigate selected microbes and, by an in deep characterization, to discover potential next-generation probiotics (NGPs) that can exert health benefits and satisfied the World Health Organization (WHO) guidelines in terms of safety, functionality, and technological usability.

Diversità fenotipica e genotipica tra potenziali probiotici di nuova generazione

La modulazione del microbiota intestinale per la gestione e la prevenzione di molte patologie è un argomento di ricerca molto attuale. In questo contesto vengono spesso utilizzati i probiotici definiti tradizionali, principalmente Lattobacilli e Bifidobatteri. Questo progetto di tesi di dottorato mira a isolare e caratterizzare in modo specifico e mirato ceppi di microorganismi probiotici di nuova generazione, selezionati e identificati per la loro potenziale capacità di apportare benefici per la salute umana e soddisfare i criteri di sicurezza, funzionalità ed uso tecnologico stabiliti dall'Organizzazione Mondiale della Sanità.

1. State-of-the-Art

During the last years, the human gut microbiota has been appreciated as a pivotal reservoir of microorganisms present predominantly in the colon - bacteria, archaea, viruses, fungi, and others - that can influence health and disease. The gut microbiota possesses different functions: digestion and metabolism of dietary elements into bioactive food components, vitamin synthesis, protection from pathogen colonization by adhering to the mucosal surface, production of antimicrobial substances, and stimulation of the immune system.
Dysbiosis is the loss of the balance among microbes, their function, distribution, or metabolic activity. This phenomenon has been linked to the development of inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), atopic asthma, allergy, obesity, type 2 diabetes (T2D), cardiovascular and neurodegenerative diseases, behavioral disorders, autoimmunity, and cancer (Durack and Lynch, 2019).
Strategies to rebalance these harmful conditions are urgently needed, such as probiotic administration, largely used nowadays. Probiotics are live microorganisms that confer a health benefit when consumed in adequate amounts, as reported by the World Health Organization (WHO) in 2002 (Hill *et al*, 2014). However, the administration of traditional probiotics is not always sufficient in specific conditions.
In silico analyses has recently increased the knowledge on microorganisms with potential health benefit to develop probiotics addressing specific consumer needs and issues. These microorganisms are referred to as Next-Generation Probiotics (NGPs) and are considered as a health promoting strategy to re-establish an eubiosis condition (Langella *et al,* 2019). Contrary to traditional probiotics, they do not have a long history of use and their safety is thus not considered as proven. These NGPs are classified as novel foods, increasing the number of requirements to reach their commercialization as food ingredients. In addition, most NGPs are currently not commercially available, and more studies are needed to address their safety, efficacy, and technological robustness. Candidates for NGPs are *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Bacteroides fragilis*, *Clostridium butyricum*, *Prevotella copri*, *Parabacteroides glodsteinii*, *Christensenella minuta*. Among these potential NGPs, *A. muciniphila* is one of the most promising microorganisms with probiotic properties (Derrien *et al*, 2004). *A. muciniphila* is a commensal bacterium that colonizes the intestinal mucosal layer, the only member of the phylum Verrucomicrobia present in the gut of healthy individuals. It is a Gram-negative, non-motile anaerobic microorganism that tolerates low oxygen levels, and produces no endospores. It degrades mucin and is also capable to induce its production by increasing the number and density of goblet cells. Its main positive functions for the host are the ability to strengthen the gut barrier, modulate insulin resistance, protect from metabolic inflammation, and exert anti-inflammatory effects (Si *et al*, 2022). Low levels of *A. muciniphila* has been associated with several diseases in both mouse models and in humans, such as IBD, UC, and Crohn's disease. However, there are many controversial aspects that need to be clarified. For example, multiple sclerosis and Parkinson's disease patients exhibited a higher abundance of *A. muciniphila* with respect to controls. The pasteurized *A. muciniphila* MucT has proven to be more efficient than the live microorganism (Cani *et al*, 2022) and at the moment the European Food Safety Authority (EFSA) has approved only pasteurized *A. muciniphila*

ATCC BAA-835^T strain as a novel food (https://www.efsa.europa.eu/en/efsajournal/pub/6780).

2. PhD Thesis Objectives and Milestones

The aim of this project is to isolate and characterize new potential NGPs investigating their crosstalk with the host, the cross-feeding processes among them, their function and mechanisms of action, their properties against intestinal pathogen colonization, their effectors responsible of the beneficial effects, also using omics and bioinformatic approaches. The results obtained with this research will deepen the knowledge about the molecular mechanisms of action of probiotics to achieve personalized treatments for the general population and for specific categories of patients.

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities (work packages, WPs) according to the Gantt diagram given in Table 1:

WP1) **Isolation of bacterial strains from fecal human samples**

Starting from stool samples from healthy donors (self-collected in sterile screw-cap specimen cups, transported refrigerated to the laboratory and processed in the same day), microorganisms of interest will be isolated (according to Filardi *et al*, 2022)

WP2) **Taxonomy identification of the isolates and genotypic characterization**

The isolates will be identified unambiguously at genus and species level by 16S rRNA gene sequencing analysis and whole genome sequencing.

WP3) **Phenotypic characterization of the new strains**

This WP3 will include different tasks (T). Indeed, the new potential NGPs will be evaluated for the macroscopic and microscopic morphological properties (T1), the tolerance to gastrointestinal conditions (T2), the ability to assimilate and/or ferment different carbon and nitrogen sources (T3), the capacity to hydrolyze compounds such as starch, lipids or proteins (T4), the production of extracellular compounds (T5), and the antibiotic susceptibility (T6).

WP4) ***In vitro* and/or *ex vivo* efficacy evaluation of the new strains**

This WP will include different tasks. Indeed, the most suitable *in vitro* and/or *ex vivo* 2D and 3D models will be used to determine the efficacy of the new potential NGPs in pathogen inhibition (T1), adhesion to eukaryotic cells (e.g., Caco-2) (T2), effect on intestinal permeability and tight junction integrity (T3), probiotic metabolite effect on different kind of cells (intestinal, endothelial and neuronal cells, adipocytes, or others) (T4).

Table 1. *Gantt diagram for this PhD thesis project.*

	1st semester	*2nd semester*	*3rd semester*	*4th semester*	*5th semester*	*6th semester*
***WP1** Isolation of bacterial strains from human fecal samples*						
***WP2** Taxonomy identification of the isolates and genotypic characterization*						
***WP3** Phenotypic characterization of the new strains*						
***WP4** In vitro and/or ex vivo efficacy evaluation of the new strains*						
Manuscript(s) and PhD thesis preparation						

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Innovative technologies to design novel and functional foods from agro-food wastes

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Co-tutor: Dr. Francesco Esposito

This PhD thesis research project aims to bioprocess agro-food wastes to obtain health-promoting bioactive compounds and organic acids through fermentation and extraction processes, supported by bioaccessibility assessment and eventually improvement techniques for the design of novel and functional foods.

Tecnologie innovative per la realizzazione di novel foods e alimenti funzionali da scarti di produzione di varie filiere agroalimentari.

Questo progetto di ricerca per la tesi di dottorato mira a biotrasformare gli scarti della filiera agroalimentare per ottenere composti bioattivi che promuovono la salute e acidi organici attraverso processi di fermentazione ed estrazione, supportati da tecniche di valutazione ed eventualmente miglioramento della bioaccessibilità per la progettazione di novel food.

1. State-of-the-Art

The agro-food industry produces a huge volume of different wastes such as coffee, nuts, beer, tomato, marine waste etc. with relevant disposal costs and environmental impact. Therefore, reintroducing wastes back into the food and non-food supply chain based on a sustainable circular economy approach belongs to the challenges of recent years. Some biotechnological and chemical techniques have been proposed as methods for waste recovery. Among these, fermentation processes have long been recognized for their potential to transform and enhance the quality of certain food matrices through microbial metabolism. Submerged fermentation (SmF) and solid-state fermentation (SSF) are two techniques used for cultivating microorganisms in a liquid media or solid media with little to no presence of water (Subramaniyam & Vimala, 2012). Both SmF and SSF have demonstrated to produce chemical compounds of industrial interest, although the latter has received greater interest due to its better efficiency in terms of cost and production. Several food matrices have been "optimized" by increasing nutritional quality or used as fermentation substrates producing chemical compounds of industrial interest such as ethanol, enzymes, organic acid, secondary metabolites etc. (Martins et al., 2011). However, some microorganisms could produce toxic compounds (e.g. mycotoxins) motivating the need to apply mitigation techniques in order to ensure product safety (Zhang et al., 2021).
Instead, some modern (and green) extraction techniques have made it possible to obtain extracts with higher specificity and yields (Lefebvre et al., 2021). Therefore, the application of some green techniques could improve the fate of fermented products. Furthermore, in vitro digestion models and encapsulation processes enable the estimation and enhancement of the bioaccessibility of some compounds. Consequently, this PhD research project aims to apply the aforementioned techniques in designing functional and novel foods, namely any food (and food ingredient) that was not consumed in significant quantities prior to 15 May 1997, as defined by Regulation (EU) 2015/2283.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) **Literature review** to identify the wastes and microorganisms of potential interest.
A2) **Characterization of agro-food waste** to determine the chemical profile **and screening of microorganisms** able to grow on the matrix.
A3) **Tuning of fermentation and chemical analysis** to assess the production of wanted (and unwanted) molecules.
A3) **Yields optimization** to higher production and recovery of the compounds with a low environmental and economic impact on industrial scale.
A5) **Product development** by selecting the formulation that provides better benefits.
A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Literature review***																								
	1) Wastes selection																								
	2) Microorganisms selection																								
A2)	***Waste and microorganism analysis***																								
	1) Chemical waste characterization																								
	2) Screening of the microorganism																								
A3)	***Tuning process***																								
	1) Fermentation trials																								
	2) Chemical analysis																								
A4)	***Yields optimization***																								
	1) Process optimization																								
	2) Chemical extraction																								
A5)	***Product development***																								
	1) post-intake behaviour																								
	2) formulation																								
A6)	***Thesis and Paper Preparation***																								

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Influence of cocoa origin and roasting parameters on the physico-chemical properties of cocoa beans and liquor

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The main objective of this PhD project is to evaluate the influence of cocoa bean origin (variety and production area) and roasting parameters (temperature, time, air flow) on the physico-chemical and sensory profiles of cocoa beans and liquor in order to find production markers useful for correlation with beans and the quality of liquor.

Influenza della origine del cacao e dei parametri di tostatura sulle proprietà fisico-chimiche di fave e paste di cacao

Lo scopo di questo progetto di dottorato è quello di valutare l'effetto dell'origine delle fave di cacao (varietà e area di produzione) e dei parametri di tostatura (temperatura, tempo e flusso dell'aria) sul profilo fisico-chimico e sensoriale delle fave di cacao e della pasta al fine di individuare dei marker correlabili con la qualità finale di questi prodotti.

1. State-of-the-Art

Physico-chemical and sensory characteristics of chocolate depend on a large number of production phases such as the origins of cocoa beans, post-harvest practices, including fermentation and drying, roasting process that transforms the molecules known as aroma precursor, which are generated during fermentation by proteolysis of the proteins stored inside this bean (Janek *et al.,* 2016), milling, tempering and so on. During roasting, flavour precursors generated during fermentation interact to produce the desired chocolate flavour (Ramli *et al.,* 2008) then an efficient roasting phase is essential for the optimal production of aromatic volatile compounds in the final product.
Roasting can also affect several cocoa bean properties such as rheological, physico-chemical and sensory ones and afterwards selecting the most suitable roasting parameters is fundamental for the quality of the chocolate but also of cocoa liquor (Swiechowski, 1996). The knowledge of the relationship between cocoa beans' origin, roasting parameters, physico-chemical and sensory profiles of cocoa beans and cocoa liquor are crucial in order to maximize the quality of final products. Several studies have shown in fact that temperature and roasting time significantly influence the rheological, chemical, sensory and nutritional characteristics of chocolate (Farah *et al.,* 2012) but also the characteristics of cocoa liquor can be influenced by the same production parameters. For example, it is possible to preserve the polyphenolic profile and obtain a liquor and a chocolate with a high nutritional value selecting appropriate roasting parameters (Zyzelewicz *et al.,* 2018).
Subsequently, the main objective of this PhD project is to evaluate the influence of cocoa bean origin (variety and production area) and roasting parameters (temperature, time, air flow) on the physico-chemical and sensory profiles of cocoa beans and liquor in order to find production markers useful for correlation with beans and liquor quality.

2. PhD Thesis Objectives and Milestones

In particular, this research work will be organized into following activities:

A1) Cocoa beans of different varieties (Forastero, Trinitario, Criollo) from different production area will be obtained from local manufacturers. Before roasting, rheological, physicochemical parameters as moisture, water activity, pH, total acidity, color (L* a* b* values), total antioxidant capacity by ABTS, DPPH and FRAP assays, total phenolic content (TPC), volatile compounds, sugars and acidic profile will be assessed.

A2) After this characterization the cocoa beans will be roasted by applying different process parameters. In particular, the cocoa beans will be roasted at four different temperatures (from 110 °C to 150 °C), four different time (from 15 to 45 min.) and two different air velocity (from 1 to 0.5 m/s). A Central Composite Design will be used in order to evaluate the effect of each parameter and their combinations.

A3) Rheological behavior of unroasted and roasted samples obtained by applying different process parameters will be studied by means of rheological empirical-imitative analysis (using a Texture Analyser) to find rheological marker related to origin and roasting parameters. Additionally, polyphenol and fatty acid profile, antioxidant capacity (DPPH and ABTS), volatile components (by using gas-chromatography coupled to a mass

spectrometry) will be evaluated on cocoa beans, to find also correlation with their origin and roasting parameters.

A4) Production of cocoa liquor using cocoa beans of different origins and undergone to different roasting treatments. Cocoa liquor will be obtained at laboratory level by using pilot equipment.

A5) Rheological, physico-chemical and sensory characterization of these cocoa liquors will be performed in order to find markers related to cocoa origin and roasting process.

A6) Writing and editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity/ Months		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
A1)	***Physico-chemical characterization of unroasted cocoa beans***																								
A2)	***Roasting processes of cocoa beans according to a CCD using different roasting parameters***																								
A3)	***Physico-chemical characterization of roasted cocoa beans***																								
A4)	***Production of cocoa liquor using pilot plant***																								
A5)	***Rheological, physico-chemical and sensory characterization of cocoa liquor***																								
A6)	***Data analysis, manuscripts and thesis preparation***																								

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AGRI-FOOD INNOVATION: STUDY OF THE CONSUMER'S PERCEPTION OF MONETARY VALUE

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This PhD thesis research project is aimed at investigating the world of innovations in the agrifood sector, evaluating: the impact of the political tools thought to advance the implementation of the innovation in the firms and the effects that this implementation has on the market value of the innovative products or realized by the innovation process, measuring the consumers' willingness to pay.

INNOVAZIONE AGRO-ALIMENTARE: STUDIO DELLA PERCEZIONE DEL VALORE MONETARIO DA PARTE DEL CONSUMATORE

L'obiettivo del presente progetto di ricerca consiste nell'indagare il mondo delle innovazioni del settore agro-alimentare, valutando: l'impatto degli strumenti politici pensati per favorire l'implementazione dell'innovazione nelle aziende e l'effetto che tale implementazione ha sul valore di mercato dei prodotti innovativi o derivanti da un processo di innovazione, misurando la disponibilità a pagare dei consumatori a riguardo.

1. State-of-the-Art

The Oslo Manual 2018, titled "The Measurement of Scientific, Technological and Innovation Activities" and written by the Organization for Economic Cooperation and Development (OECD), defines the term "innovation" as "a new or improved product or process (or combination thereof) that significantly differs from the unit's previous products or processes and has been made available to potential users (product) or brought into use by the unit (process)". The term "unit" is used to describe the actor responsible for innovations. Politics, research, business, and consumers collectively play a role in this innovation process. Politics funds public and private research projects, which businesses implement based on consumers' demand. It is crucial that the relationships within this network are efficient and effective in order to achieve the sustainable development goals outlined in the Farm to Fork Strategy and the European Green Deal.

The growing interest in agri-food innovation has led to an inevitable increase in scientific publications on this topic, covering both innovative proposals and the assessment of innovative case studies. A bibliographic research was conducted to understand the current research trends in order to write a systematic literature review on the methods, tools, and theories used to evaluate innovation in the agri-food sector. The keywords "agricultural" OR "agri-food" AND "innovation" AND "assessment" OR "evaluation" OR "measurement" were entered into the Scopus database. Out of the 1185 results, 116 were deemed suitable for addressing the research question.

Several methodologies have been applied, with Life Cycle Assessment (Verdi et al., 2022; Vaglia et al., 2022; Stillitano et al., 2019) being the most commonly used. Many studies focus on evaluating the social, economic, and environmental impacts of innovations. Other examples include cost-benefit analysis and the Material Circularity Indicator (MCI) (Falcone et al., 2022) for evaluating circular economy aspects, Living Labs, SWOT analysis, and the Digital Economy and Society Index (Metta et al., 2022) for measuring responsible digitization in agriculture, and the Adjusted Food Sustainability Index, Policy Index, and Data Envelopment Analysis (Agovino et al., 2018) for evaluating policy efficiency in the field of food sustainability. These are just a few examples.

Therefore, although the initial plan was to evaluate the added value of innovation in the market through an analysis of willingness to pay, the best methodology, among those identified in the bibliographic analysis, will be developed by considering multiple variables such as research costs, time, the object of research, and the perspectives of the actors seeking to introduce the innovation.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Definition of the scope of innovation**. Due to the complex nature of the phenomenon, it is important to determine, based on the results of the bibliographic review, whether to develop the analysis considering: the entire agri-food sector, a specific supply chain or a precise phase of the supply chain (A1.1). Furthermore, the

focus could be on product, process, or system innovation (A1.2), and the analysis may involve all actors of innovation, a specific actor, or a subset of actors (differentiating between supply and demand) (A1.3).

A2) **Identification of the assessment method**. The appropriate tools will be acquired (A2.1) and the relevant data will be collected (A2.2) in accordance with the prescribed methodology and the actors under investigation.

A3) **Application of the chosen method** to produce statistically significant data.

A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Definition of the scope of innovation***																								
	1) choice of the level of detail																								
	2) choice of kind of innovation																								
	3) choice of the actors																								
A2)	***Assessment method***																								
	1) adoption of tools																								
	2) data collection																								
A3)	***Application of the method***																								
A4)	***Thesis and Paper Preparation***																								

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Analysis of volatilome of olive oils and flavoured oils: quality grade evaluation and study of modification during storage

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The main aims of this research project are: i) setting up of chemometrics models based on volatilome analysis by HS-GC-IMS to predict olive oil commercial category, ii) evaluation of the qualitative and quantitative modification of volatile profiles of olive oils and flavoured oils during storage time. A total of 150 olive oil samples will be sensory evaluated and the volatile organic compounds of the same oils will be analyzed by HS-GC-IMS technique. The volatilome of a selected set of olive oils and flavoured oils will be studied by SPME-GC-MS during the storage time to register possible changes.

Analisi del volatiloma di oli di oliva e oli aromatizzati: valutazione della categoria merceologica e studio delle variazioni in conservazione

I principali obiettivi di questo progetto di ricerca sono: i) messa a punto di modelli chemiometrici per stimare la categoria merceologica dell'olio di oliva, ii) valutazione delle modifiche qualitative e quantitative dei profili in composti volatili di oli di oliva e di oli aromatizzati durante la conservazione. Un totale di 150 campioni saranno valutati sensorialmente ed i composti volatili degli stessi oli saranno analizzati mediante la tecnica HS-GC-IMS. Inoltre, il volatiloma di un set selezionato di oli di oliva e di oli aromatizzati sarà studiato attraverso SPME-GC-MS per registrare i possibili cambianti durante la conservazione.

1. State-of-the-Art

In the Mediterranean area, especially in Spain, Italy and Greece, olive oil represents one of the main food products with a world production of 2,511,000 tonnes expected for the 2022/23 campaign (DG AGRI, 2023). In the European Union (EU), virgin olive oils (VOOs) can be classified into three commercial categories, based on both physicochemical and sensory parameters, such as: extra virgin (EV), virgin (V) and lampante (L) (Reg. EC n. 2022/2104). Despite several modifications that occurred over the years, the official method for sensory evaluation still shows some weaknesses as it is time-consuming and, in case of a non-correct training of assessors (Barbieri *et al*., 2020), can be affected by not satisfactory reproducibility of results. For this reason, the identification and quantification of volatile organic compounds (VOCs) in VOOs is of great importance for assessing their quality. In fact, targeted and untargeted instrumental methods, based on the analysis of such molecules, can be considered as an interesting tool useful to support the Panel test (Cavalli *et al*., 2004). Specifically, some VOCs have been proposed as markers to detect positive (e.g., fruity) and negative sensory attributes according to their concentrations (Valli *et al*., 2020). For this purpose, targeted methods based on headspace solid phase microextraction (SPME) with the use of flame ionization detector (FID) or mass spectrometry (MS) are being recently validated (Aparicio-Ruiz *et al*., 2023). Furthermore, rapid instrumental methods concerning gas-chromatographic techniques, such as Flash-GC and Ion Mobility Spectrometry (HS-GC-IMS) can be also useful for this aim permitting a fast pre-classification of samples and increasing the efficiency of quality control analyses (Valli *et al*., 2020). Finally, in the global economic scenario of olive oil, flavoured oils are becoming increasingly popular. Customers are attracted by their versatility of culinary use due to the possibility to convey a wide range of aromas to food preparations thanks to the use of different kind of flavouring matrices as herbs, spices, fruits, and vegetables (Baiano *et al*., 2016). The addition of specific flavouring agents to olive oils, depending on the applied technology to produce the flavoured oil (co-extraction, contact and essential oils inclusion), affects the incorporation in the oil matrix of specific bioactive compounds with antioxidant and/or healthy and/or sensory properties. Consequently, the analytical assessment of flavoured oils taking into consideration compositional and sensory characteristics is essential to check the product quality and to study the performance during storage (Lamas *et al*., 2022).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Sampling**: preparation, anonymization, and shipment of oils to the involved laboratories.

A2) **Definition of instrumental and sensory protocols:** setting up of analytical protocols for GC-IMS and

sensory analysis to be applied in a shared mode by the five different laboratories.

A3) **Sensory and instrumental alignment tests**: verification of the degree of analytical alignment of five GC-IMS instruments using specific standards prepared ad hoc. The same analytical protocol has to be applied by the five laboratories participating in the trial (A3.1); check of sensory alignment among the five panels participating in the trial by application of a specific decision tree scheme (A3.2).

A4) **Creation of sensory and instrumental datasets:** the dataset will consist of at least 150 samples analysed by both sensory and instrumental analysis (GC-IMS).

A5) **Development of chemometric models:** estimation models (EV vs V) will be built using the dataset (A4).

A6) **Shelf-life study**: evaluation of the sensory characteristics and volatile profiles of selected samples (EV and V olive oils) monitored during the shelf-life (0, 6, 12 months) by SPME-GC-MS/FID and sensory descriptive analysis.

A7) **Volatile and sensory analysis of flavoured oils**: flavoured oils of particular interest for the company will be selected; volatilome and sensory characteristics as well as modification during storage will be monitored.

A8) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Sampling***																								
A2)	***Definition of instrumental and sensory protocols***																								
A3)	***Sensory and instrumental alignment tests***																								
	1) Instrumental alignment																								
	2) Sensory alignment																								
A4)	***Creation of sensory and instrumental datasets***																								
A5)	***Development of chemometric models***																								
A6)	***Shelf-life study***																								
A7)	***Volatile analysis of flavoured oils***																								
A8)	***Writing and Editing***																								

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Functional Screening of Microbial Resources for Healthy Food Fermentations through a Predictive Understanding of Genotype-Phenotype Relationships

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Increasing concerns about human and planetary health has raised the need for healthier and more sustainable food alternatives mainly obtained from plant sources. In this context, traditional fermentation offers a valuable biotechnological approach to maintain and/or improve plant-based foods nutritional, techno-functional and sensory properties. Accordingly, this Ph.D. research project aims to evaluate the suitability of different microbial resources to be employed for successful plant-based fermentation, by comparing their fermentative, phenotype, and metabolic capabilities when cultivated under optimal and/or food-like conditions. The most promising strains will be selected to produce three newly developed fermented plant-based food prototypes potentially having outstanding quality.

Screening funzionale di risorse microbiche per fermentazioni alimentari salutari attraverso un'analisi predittiva di relazioni genotipo-fenotipo

Le crescenti preoccupazioni per la salute umana e planetaria hanno evidenziato la necessità di alternative alimentari più sane e sostenibili, ricavabili principalmente da fonti vegetali. A questo proposito, la fermentazione offre un valido approccio biotecnologico per mantenere e/o migliorare proprietà nutrizionali, tecno-funzionali e sensoriali di alimenti vegetali. In tal senso, questo progetto di dottorato mira a valutare diverse risorse microbiche come potenziali starter per una fermentazione funzionale di substrati vegetali, confrontandone capacità fermentative, fenotipiche e metaboliche in condizioni di coltivazione ottimali e/o food-like. I ceppi più promettenti saranno impiegati nello sviluppo di prototipi di alimenti vegetali fermentati di migliorata qualità.

1. State-of-the-Art

There is an increasing need in society, and in industry, for a revolutionary change in the food system to a more sustainable dietary pattern richer on plant-based foods (*Graça et al., 2019)*. Although a large portion of consumers has already started to follow this predominately plant-based lifestyle, due to healthy (i.e., lactose intolerance, diabetes, cardiovascular diseases and others), ethical (i.e., vegetarianism and/or veganism) or environmental reasons, still there is a part of the population that is resistant to undergo this transition process and overconsumes meat, dairy (and ultra-processed) foods and beverages (*Graça et al., 2019*). Interest in non-boring and tasteful alternative protein sources to replace the traditional animal-protein rich sources (i.e., dairy and meat) is thriving, leading to a continuous introduction of traditional and new sources of plant-based proteins, particularly from cereals, pseudo-cereals, and legumes (*Rizzello et al., 2010*). Indeed, their nutritional and functional properties make these sources particularly interesting alternative to the counterpart of animal origin (*Coda et al., 2017*). Despite being strongly recommended for human diet, plant-based protein sources have several limitations such as high anti-nutritional compounds content and lack of some essential amino acids (C*oda et al., 2017*). The fortification of cereal-based raw materials with legumes may represent a good strategy to complement the product nutritional quality (C*oda et al., 2017*). From a technological point of view, the interaction between the proteins and the carbohydrates present in grain- (and/or legume-) based raw materials may negatively interfere with foaming, gelling and other techno-functional properties. From a sensory point of view, bitter and beany (especially legumes) taste can form due to lipid oxidation causing bad taste and off-flavor (*Rizzello et al., 2010*). For these reasons, biotechnological approaches are required. Indeed, fermentation has been used for millennia for food preservation (and shelf-life increasing) and for enhancing food flavor. Recently, fermentation has become a simple and valuable biotechnology to keep and/or enhance the nutritional, textural, sensory properties of a raw material (*Di Cagno et al., 2013*). In this context, fermentation may offer a possible solution to overcome most of the challenges related to the application of high-protein plant-based raw materials in food production (*Coda et al., 2017*). From a nutritional point of view, fermentation may allow to improve protein digestibility, to reduce anti-nutritional factors and metabolize nutritional constituents (i.e., phenolic compounds) (*Coda et al., 2017*).
From a technological point of view, fermentation may improve foaming and emulsifying properties (*Coda et al., 2017*). Exopolysaccharides production by microorganisms can further enhance raw textural/structural properties (*Gobbetti et al., 2014*) while favoring the adhesion and the persistence of microbial cells, introduced in human body via consumption of probiotics and/or food containing living microorganisms (i.e., yogurt), at the intestinal

epithelium level (*Gobbetti et al., 2014*). Finally, fermentation can be used to improve food flavor and sensory characteristics (*Gobbetti et al., 2014*). Facing this background, the following research project aims to explore how different microbiological resources (mainly lactic acid bacteria (LAB) including, fructophilic lactic acid bacteria (FLAB), and yeasts) can be fully exploited to improve traditional and/or develop innovative fermented plant-based (cereals and/or legume based) food, rich in proteins, with an enhanced nutritional, textural and sensory value, and with health-promoting potential.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Literature Review Experimental and Plan Definition**

A2) **(*Existing*) microbial resource(s)** will be characterized for desired functional activities when individually cultivated in optimal and/or in food-like conditions (A2.1). Eventual differences in strains phenotypic profiling will be highlighted by OmniLog® Phenotype MicroArray (PM) platform (Biolog System) (A2.1). To the same purpose, autochthonous microbial resources will also be newly isolated and identified (A2.2).

A3) **Raw materials (legume- and/or grain- flours)** will be evaluated alone or in different combinations for their performances during prototypes production (A3.1). The best raw materials combination, identified during activity A3.1, will be used as a substrate for fermentation in activity A3.2.

A4) **Fermented prototype(s)** will be developed and characterized for their nutritional, techno-functional, and sensory properties, and for their possible effect on human gut (A4.1). The better-performing food prototype(s), from activity A4.1, will be selected and considered in the setting up of an optimized fermentation protocol to be applied at industrial level (A4.2).

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1. Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Literature Review and Experimental Plan Definition***																								
A2)	***Microbial Resource(s)***																								
	1) Characterization of *existing* microbial resources																								
	2) Isolation and identification of *novel* microbial resources																								
A3)	***Raw Material(s)***																								
	1) Evaluation of raw materials (legume- and/or grain- flours)																								
	2) Fermentation of raw materials (legume- and/or grain- flours)																								
A4)	***Fermented Prototype(s)***																								
	1) Development, characterization, and optimization of fermented prototype(s)																								
	2) Upscaling of fermented prototype(s) production																								
A5)	***Thesis and Paper Preparation***																								

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Valorisation of dairy production in inland areas: product and process innovation

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The project aims at innovating the realization of dairy products in the inland areas of Molise, respecting their tradition. After a careful analysis of raw materials and production processes, innovative products will be developed using ingredients, also from the territory, containing bioactive compounds, to increase their health/nutritional value.

Valorizzazione delle produzioni lattiero-casearie delle aree interne: innovazione di prodotto e di processo

Il progetto ha come obiettivo l'innovazione delle produzioni lattiero-casearie delle aree interne del Molise, nel rispetto della loro tradizione. Dopo un'analisi accurata delle materie prime e dei processi di produzione, verranno sviluppati prodotti innovativi mediante l'utilizzo di ingredienti, anche provenienti dal territorio, contenenti composti bioattivi, al fine di incrementarne il valore salutistico/nutrizionale.

1. State-of-the-Art

The proposed research topic is the enhancement of dairy production in inner areas of Molise. In this regional economy, the dairy sector represents one of the most consolidated ones, perfectly connected with the artisan dairy tradition and the consistency of the dairy cattle herd (about 12500 heads in 2020). Due to the link of the supply chain to the production, the Molise dairy system is based on the model of typical productions, whose specificity is the result of multiple factors. The "typicality" factor tends to be inherent in the origin of the product, or rather, in the presence of a link between product and territory, which is recognized in the origin of the raw material and/or in the location of the processing activities. However, strengths, represented by tradition, have to face difficulties in the agricultural conversion, in improving the quality of products and/or innovation in well-defined sectors and the empiricism of traditional technologies. The collaboration between the world of research and the industrial production led to a product/process innovation that allowed having food that combine the right quality/price ratio and high nutritional standards, in safety. It should be added that the traditional agri-food products are evolving towards new proposals (packaging, service, characterization) to meet the new needs of consumers. For example, innovative biodegradable packaging/coatings, especially those carrying antimicrobial agents, were proved to extend shelf life of cheese without having a negative impact on sensory properties (Jafarzadeh *et al*, 2021). Re-evaluating and promoting the traditional productions, through targeted research actions adapted to the size of the company, becomes very important to answer to the new needs of the European market and, at the same time, support the competitive advantage of these productions. The PhD project aims at broadening the qualitative dimension of the company productions, through technological and organizational transformations, fully consistent with the development objectives of the "National Strategy for the Inner Areas of the Country" (National Strategy for Inner Areas, 2013). The project proposal aims at increasing the competitiveness of the dairy sector of the inland areas of the Alto Medio Sannio. This will be done through the valorisation of the local dairy productions and the development of tools and methodologies, also characterized by innovation, able to ensure the enhancement of the quality and safety of dairy products of excellence, with a clear territorial identity, and to promote a policy of synergy and efficiency of the entire agri-food chain. For example, adding ingredients from the agriculture and the territory, rich in healthy compounds, could lead to an improvement of the nutritional value of the dairy products, encouraging the circular economy and generating a sustainable and healthy production (Picciotti *et al*, 2022). Another way to innovate could be the use of milk from different origin. The development of appropriate technologies for the production of innovative cheeses, using a mixture of cow and ewe or goat milk, could be an interesting and feasible opportunity for the dairy industry (Niro *et al*, 2014). Traditional dairy productions, if appropriately enhanced/innovated in compliance with the needs of the market and the consumers, can play an important social role, helping to maintain not only stable traditions, but also farms, rural population and local production, processing, marketing and enogastronomic tourism companies.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities, according to the Gantt diagram given in Table 1:

A1) **Bibliographic research** and evaluation of the state of the art.

A2) **Characterization of pastures/forages, starting milk and products,** through chemical-nutritional and technological analysis to evaluate the quality of raw materials and products (A2.1) and *shelf-life* tests (A2.2) to determine the durability of the dairy products. Study/evaluation of product/process indicators.

A3) **Evaluation/optimization of the processes and packaging**, through the evaluation/optimization of process parameters (A3.1) and new packaging methods (A3.2) that could improve the durability of the products. Study/evaluation of product/process indicators.

A4) **Development of traditional/innovative products** with new formulations that can include ingredients from the territory (A4.1) and evaluation/optimization of process parameters to obtain the innovative dairy products (A4.2). Study/evaluation of product/process indicators.

A5) **Characterization of innovative traditional products,** through chemical, physical, sensorial, safety of use, nutritional and functional evaluation (A5.1) and *shelf- life* tests (A5.2) to determine the durability of the innovative products. Study/evaluation of product/process indicators.

A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months		2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1)	***Bibliographic research***																		
A2)	***Characterization of pastures/ forages/ starting milk and products***																		
	1) Chemical-nutritional and technological analysis																		
	2) *Shelf-life* tests																		
A3)	***Evaluation/optimization of the processes and packaging***																		
	1) Evaluation of process parameters																		
	2) Evaluation of new packaging methods																		
A4)	***Development of traditional/innovative products***																		
	1) Formulations of innovative products																		
	2) Optimization of processes																		
A5)	***Characterization of innovative made products***																		
	1) Chemical-physical, sensory, sanitary, nutritional and functional evaluation																		
	2) *Shelf-life* test																		
A6)	***Thesis and Paper Preparation***																		

3. Selected References

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Inhibition of Meat Fat Autoxidation by Natural Essential Oils

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Co-Supervisor: Prof. Giovanna Ferrentino

During storage, lipid oxidation deteriorates Speck meat fat quality. To inhibit oxidation, producers use synthetic antioxidants. However, this research will investigate essentials oils to preserve meat fat. Several officinal herbs and spices will be extracted by supercritical carbon dioxide extraction and tested for their capacity to inhibit meat fat oxidation. Soxhlet method will be used for quantifying the total fat content, and NMR to analyze the content of fatty acids. A Kinetic-based DPPH assay will be performed to understand mechanism of the antioxidant's activity. Also, isothermal calorimetry and oximetry will be extensively used to measure the oxidizability of meat fats.

Inibizione dell'autossidazione dei grassi di carne da parte degli oli essenziali naturali

Durante lo stoccaggio, l'ossidazione lipidica deteriora la qualità del grasso della carne di Speck. Per inibire l'ossidazione, i produttori usano antiossidanti sintetici. Tuttavia, questa ricerca studierà gli oli essenziali per preservare il grasso della carne. Diverse erbe e spezie officinali saranno estratte mediante estrazione di anidride carbonica supercritica e testate per la loro capacità di inibire l'ossidazione del grasso della carne. Il metodo Soxhlet sarà utilizzato per quantificare il contenuto totale di grassi e NMR per analizzare il contenuto di acidi grassi. Verrà eseguito un test DPPH a base cinetica per comprendere il meccanismo dell'attività dell'antiossidante. Inoltre, la calorimetria isotermica e l'ossimetria saranno ampiamente utilizzate per misurare l'ossidabilità dei grassi della carne.

1. State-of-the-Art

1.1 Auto-oxidation in meat products

Meat and meat products are integral to the human diet and are an essential source of minerals, vitamins, and many other essential nutrients (Zhang, Xiao *et al*. 2010). Meat fat is the main target of oxidation, as it contains high levels of unsaturated fatty acids. The oxidation of meat fat can be accelerated by several factors such as heat, light, and oxygen (Chen, Zhou *et al*. 2015). Adding natural antioxidants and essential oils to meat can help delay the oxidation process and increase the shelf life of meat products.

According to a study, inclusion of BHA and BHT increased the oxidative stability of speck (Neethling, *Suman et al.* 2016), but these synthetic antioxidants are not very beneficial for health and these days trend is more towards using antioxidants driven from natural sources like plants extract herbs and spices.

1.2 Role of essential oils in meat fat stability:

Essential oils are also being researched for their potential to act as natural antioxidants in meat products. According to studies, essential oils can effectively stop meat oxidation and extend its shelf life (Rojas and Brewer *et al.* 2007). To get the highest antioxidant activity, the ideal quantities and combinations of essential oils must be determined.

1.3 Experimental methodologies to analyze meat oxidation and stability:

Fat extraction using soxhlet method offers a repeatable and precise determination. NMR technique will be used due to its excellent sensitivity and selectivity for both saturated and unsaturated fatty acids (Marcone, Wang *et al.* 2013). To determine whether the antioxidant mechanism of speck fat is based on hydrogen atom transfer or electron transfer, kinetic-based DPPH test will be carried out in a variety of solvent systems. Oximetry will be used to monitors the oxygen intake or consumption during chemical processes, whereas isothermal calorimetry measures the heat produced or absorbed during chemical reactions (Klettenhammer, Ferrentino *et al*. 2023). A thorough knowledge of the antioxidant capabilities of essential oils can be obtained by combining these approaches. This information will aid in the creation of novel techniques for maintaining and improving the quality of meat products.

2. PhD Thesis Objectives and Milestones

The research objectives of this PhD project can be achieved through the following activities and working plan as shown in the Gantt diagram given in Table 1:

A1) **Literature review and analysis of speck meat fat oxidation:** Literature review and analyze the oxidizability of speck meat fat from South Tyrol and find the role of natural essential oils in the inhibition of speck meat fat autoxidation.

A2) **Optimization of methods:** Optimization of methods for extraction of herbs and spices using supercritical carbon dioxide extraction and analysis of meat fat oxidation using isothermal calorimetry.

A3) **Antioxidant capacity of essentials oils:** To investigate the antioxidant capacity and optimal concentration of different essential oils required to prevent oxidation of speck meat fat during storage without affecting its taste.

A4) **Comparative analysis of natural and synthetic antioxidants:** The difference between the efficacy of essential oils and synthetic antioxidants in inhibiting speck meat fat oxidation will eventually promote the use of natural essential oils to be used in preserving speck meat.

A5) **Finalization of dissertation and PhD Defense:** Finalization of the PhD thesis, scientific papers and poster communications.

Table 1 *Gantt diagram for this PhD thesis project.*

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Literature review and analysis***																								
	1) Literature Review																								
	2) Oxidation analysis using TAM																								
A2)	***Optimization of methods***																								
	1) Supercritical carbon dioxide extraction method																								
	2) Isothermal Calorimetry method																								
A3)	***Antioxidant capacity of essential oils***																								
A4)	***Comparative analysis of natural and synthetic antioxidants***																								
A5)	***Finalization of dissertation and Paper Preparation***																								

3. Selected References

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Neethling, N. E., et al. (2016) Muscle-specific colour stability of blesbok (Damaliscus pygargus phillipsi) meat. **119**: 69-79.

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Klettenhammer, S., et al. (2023) Oxidative stability by isothermal calorimetry of solid lipid microparticles produced by particles from gas saturated solutions technique. **173**: 114370

Improvement of Quality and Nutritional Value of Foods Using Natural Compounds and Mild Biotechnologies

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This PhD thesis project is aimed at developing biotechnological approaches to reformulate food products through new ingredients and recalibrate processes, especially heat treatments, also through emerging mild technologies. This goal is designed to innovate traditional foods into products with enhanced quality and safety features, prolonged shelf life, improved nutritional value, organoleptic characteristics and functional features, while reducing water and energy consumption.

Miglioramento della qualità e del valore nutrizionale degli alimenti mediante l'uso di composti naturali e processi biotecnologici "mild"

Questo progetto di tesi di dottorato è finalizzato allo sviluppo di approcci biotecnologici per riformulare i prodotti alimentari attraverso nuovi ingredienti e ricalibrare i processi, in particolare quelli termici, anche attraverso tecnologie emergenti. Questo obiettivo è pensato nell'ottica di ottenere alimenti con caratteristiche di maggiore qualità e sicurezza, prolungata conservabilità, migliore valore nutrizionale, caratteristiche organolettiche e funzionali rispetto a quelli tradizionali di riferimento, riducendo al contempo il consumo di acqua ed energia associato ai processi produttivi.

1. State-of-the-Art

Food and nutrition security and transitions to sustainable food systems are currently major topics for the agri-food sector. The increase in global population, which is projected to reach over 9 billion by 2050, in combination with climate changes, pose a serious threat to food security as arable land becomes increasingly limited. On the other hand, approximately 1.3 billion tons of food is reported to be yearly lost or wasted globally along the supply chain, i.e. through agricultural practices, postharvest handling and storage, processing, distribution and during food preparation. This results in over one-third of the food produced worldwide, while over 870 million people still suffer hungry (FAO, 2016).

In this context food processing and technology have a key role in transforming raw materials into safe foods with extended shelf life, desired nutritional properties, high quality and improved functional properties. For these goals, food industry continuously faces the need to innovate and advance the technologies currently used. This is to meet the global demands of increasing food security and assuring sustainability, while responding to the consumers changing dietary choices and request for safe, high quality and healthy foods resembling natural and fresh-like products (Tian *et al*., 2016).

The need to guarantee an adequate shelf life to foods often relies on heat treatments, which can partially impair their nutritional value, or debated preservatives which are negatively perceived by consumers. Natural alternatives can be based on ingredients derived from plants, e.g essential oils or plant extracts, through green biotechnologies also through their recovery from agri-food byproducts. It is in fact widely reported that they are characterized by bioactivities, e.g. antioxidant and antimicrobial ones, which make them valuable alternatives to traditional preservatives (Tongnuanchan and Benjakul, 2014). Moreover, bioprotective and tailored starter cultures can be used assure shelf life and safety and, eventually, to enrich foods with relevant functional and nutritional compounds, or reduce the content of antinutritional ones. Also, literature reports good synergistic action with emerging non-thermal technologies, e.g. cold plasma, high pressure, pulsed electric fields, or mild heat processes which can be a sustainable approach to innovate food processing to preserve food safety, functional, nutritional and sensory properties.

This PhD thesis project will be addressed to the development of biotechnological approaches to reformulate food products through new ingredients and recalibrate processes, especially heat treatments. This goal is aimed at innovating traditional foods into products with enhanced quality and safety features, prolonged shelf life, improved nutritional value, organoleptic characteristics and functional features.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following

activities according to the Gantt diagram given in Table 1:

A1) **Review of the scientific literature** in the field of food processing waste and by-products as a source of bioactives, and emerging non thermal technologies as alternatives to heat treatments, sanitizers and chemical preservatives in relation to different food products.

A2) **Functional characterization of the by-products:** different by-products (e.g. citrus paste, olive pomace, residues from fish, fruit and vegetable processing) will be selected and tested for some bioactivities, e.g. antioxidant, prebiotic towards some commercial probiotic bacteria, antimicrobial/antifungal against foodborne pathogenic (e.g. *L. monocytogenes, E. coli, S.* Enteritidis, *S. aureus*) and spoilage microorganisms (e.g. *Enterobacteriaceae, Pseudomonas* spp*., Bacillus* spp., yeasts and moulds). The evaluation will be done in model systems (liquid in microtiter plates, solid in Petri dishes) at different pH conditions, water activity, temperature conditions and microbial inoculum level.

A3) **Screening and selection of LAB and yeasts species** Lactic acid bacteria and yeasts newly isolated and from the Microbial Culture Collection of the University of Bologna will be screened for pro-technological and functional properties. Each strain will be inoculated in synthetic media, and the ability to produce e.g. bacteriocin, phenyllactic acid, γ-aminobutyric acid, exopolysaccharides, aroma compounds will be assessed. Growth ability and acidification when using by-products as substrate will be also evaluated.

A4) **By-products valorisation through microbial fermentation**: strains of the most promising yeasts and lactic acid bacteria will be used to ferment the by-products in order to enhance their bioactivities and use them as functional food ingredients.

A5) **Definition of experimental design** to reformulate selected food products and recalibrate the processes by exploiting the most promising biotechnological solutions outlined by the previous activities. Also the interactive effects with non-thermal treatments, e.g. cold plasma, high pressure, will be assessed to obtain foods with enhanced quality and safety features, prolonged shelf life, improved nutritional and functional features.

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Literature review and working plan***																								
A2)	***By-products characterisation***																								
A3)	***Screening and selection of LAB and yeasts strains***																								
A4)	***By-products valorisation through microbial fermentation***																								
A4)	***Food reformulation and process recalibration***																								
A5)	***Thesis and Paper Preparation***																								

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Identification of Microbial Functions Interfering with Host-Cell Physiology: a Quick View into *Streptococcus thermophilus* Metabolic Potential

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Co-tutor: Prof. Stefania Arioli

This PhD thesis research project is aimed at demonstrating how significant interactions between specific microbial activities and host can play a potentially crucial role in upgrading the health status in fragile consumers categories. Particularly, the study focuses on the modulation of *Streptococcus thermophilus* urease and β-galactosidase activities through a metabolic boost, required for the development of new functional products able to affect gastro-intestinal diseases patients' symptoms as a completely new way of treatment, *i.e.* from traditional probiotics to "precision probiotics".

Identificazione di funzioni microbiche in grado di interferire con le interazioni fisiologiche ospite-cellula: uno sguardo al potenziale metabolico di *Streptococcus thermophilus*.

Questo progetto di tesi di dottorato si pone come obbiettivo di dimostrare come alcune interazioni significative tra specifiche attività microbiche e ospite possano avere un ruolo cruciale nel *miglioramento* delle condizioni di salute nelle categorie fragili di consumatori. In particolare, lo studio si incentra sulla possibile modulazione dell'attività ureasica e β-galattosidasica di *Streptococcus thermophilus* tramite specifiche azioni di *potenziamento* metabolico. Queste attività sono funzionali allo sviluppo di nuovi prodotti in grado di ridurre i sintomi in pazienti affetti da specifiche malattie legate al tratto gastro-intestinale e rappresentano un primo passo nella transizione dai prodotti probiotici tradizionali ai "probiotici di precisione".

1. State-of-the-Art

Streptococcus thermophilus, which is employed in dairy and probiotic industry, has an efficient lactose catabolism managed first by LacS, a permease system, and then hydrolyzed by β-galactosidase to yield glucose and galactose (Arioli et al., 2022), but it also owner of an urease positive activity trait that showed a significant positive correlation with β-galactosidase, increasing lactose consumption rate and acidification potential (Mora et al., 2014). Increasing β-galactosidase activity in *S. thermophilus* could be resolutive in the development of a new product able to reduce lactose intolerance symptoms in those subjects having a limited expression or activity of lactase in the small intestine, thus suffering of discomfort and pain after dairy consumption. Lactose intolerant subjects often react by excluding milk and dairy from their diet thereby leading to essential nutrient deficiency, such as vitamin D and calcium increasing the risk of osteoporosis (Ratajczak et al., 2020). In this context a food supplement based on *S. thermophilus* metabolically activated for lactose consumption will also be inline with the claim of "lactose digestion" by yogurt cultures recognized by EFSA (EFSA Journal 2010; 8(10):1763) and with the more recent World Gastroenterology Organisation Global Guidelines – Probiotics and prebiotics (2023).
More recently it was reported that a *S. thermophilus* strain releasing β-galactosidase was able to significantly reduce tumorigenesis of colorectal cancer in mice (Li et al., 2021) by modulating the metabolism of cancer cells, *i.e.* reducing the aerobic glycolysis and activating the oxidative phosphorylation. Therefore, the possibility to prepare specific *S. thermophilus* strains to be prone in releasing β-galactosidase *in vivo* will be an issue of this PhD project. IBD (Inflammatory Bowel Disease) is a term used to describe different disorders that involve chronic inflammation of the digestive tract. IBD can manifest by various gastrointestinal expressions that share a common genesis in an initial state of dysbiosis (Strober et al., 2007) which is often related to high level of pathogenic urease activity in colon. Ammonia released by urease activity of harmful gut bacteria increases the inflammatory status in these patients. We recently observed that administration of urease-positive *S. thermophilus* cells in healthy subject determined a decrease in urease content in fecal samples (Martinović et al., 2023). Therefore, the chance to prepare *S. thermophilus* with high content of urease activity and to test them *in vivo* will also be a target of this PhD project.
Based on these, the aim of the study is to prove that an efficient modulation of *S. thermophilus* metabolic activities and an adequate administration of freeze-dried cells could be useful to host treatment by:

- Improving lactose intestinal absorption better than commercial lactase would do.
- Modulating cancer cells metabolism and decreasing tumorigenesis of colorectal cancer in mice.

- Providing a correction of dysbiosis state with a re-population by harmless bacteria and a decrease of pathogenic urease activity in the gut.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project would be performed subdivided into the following activities (A) according to the Gantt diagram given in **Table 1**:
A1) **Screening and selection** of *S. thermophilus* strains based on the level of their β-galactosidase and urease activities.
A2) **Metabolic improvement** of *S. thermophilus* strain selected in A1 to increase β-galactosidase and urease activities.
A3) **Scale-up of the metabolic improvement** from a laboratory-scale to an industrial-pilot-scale.
A4) **Optimization of the freeze-drying process** to maintain the highest level of β-galactosidase and urease activities in *S. thermophilus* cells.
A5) **Proof-of concept *in vivo* study in lactose malabsorber human subjects** to verify the efficacy of metabolically activated *S. thermophilus* freeze-dried biomasses in reducing the lactose-intolerance symptoms.
A6) **Proof-of concept *in vivo* study on C57BL/6J-ApcMin/J mice**, which harbor a germline mutation in the tumor suppressor gene Apc and develop intestinal polyps spontaneously, to verify the role of *S. thermophilus* releasing and not releasing β-galactosidase on tumorigenesis.
A7) **Proof-of concept *in vivo* study on mice** to evaluate the decrease of fecal urease activity by administering *S. thermophilus* prepared to be urease-positive or urease-negative.
A8) **Dissemination, preparation and publication** of the results of the project
A9) **Writing and editing of the PhD thesis**, scientific papers and oral and/or poster communications.

Table 1 *Gantt diagram for this PhD thesis project.*

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	*Selection of S. thermophilus strain*																								
A2)	*Metabolic improvement of S. thermophilus selected strain*																								
A3)	*Scale-up process from a laboratory scale to an industrial/pilot scale*																								
A4)	*Optimization of the freeze-drying process*																								
A5)	*Proof-of concept in-vivo study in lactose malabsorber human subjects*																								
A6)	*Proof-of concept in-vivo study on C57BL/6J-ApcMin/J mice to verify the role of S. thermophilus releasing and not releasing β-galactosidase on tumorigenesis*																								
A7)	*Proof-of concept in-vivo study on mice with administration of urease positive or urease negative S. thermophilus cells*																								
A8)	*Project's results dissemination, preparation and publication*																								
A9)	*PhD thesis writing and editing*																								

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Improving Sustainability of the Vegetable Oils Supply Chains: Innovative Analytical Methods for Quality Control, Valorization of By-products and Reduction of Waste

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This PhD thesis research project is aimed at improving the sustainability of the vegetable oils supply chains, considering the application of innovative analytical methods and the reuse of olive mill by-products and waste vegetable oils. In fact, the research is focusing on the development of innovative, rapid, as well as solvent and reagent efficient analytical approaches for oil quality control (e.g. the use of the microESR to measure oxidative state), on the valorization of mill by-products (such as olive pomace, wastewater, etc.) as raw materials for several industrial applications as well as on the reuse of waste cooking oils (e.g. frying oils) for the biodiesel production.

Miglioramento della sostenibilità delle filiere degli oli vegetali: metodi analitici innovativi per il controllo qualità, la valorizzazione di sottoprodotti e la riduzione degli sprechi

Il progetto di ricerca di tesi di dottorato mira a migliorare la sostenibilità delle filiere degli oli vegetali, considerando l'applicazione di metodi analitici innovativi e il riutilizzo di sottoprodotti oleari e oli vegetali di scarto. Infatti, le ricerche si stanno concentrando sullo sviluppo di approcci analitici innovativi, rapidi ed efficienti in termini di impiego di solventi e reagenti per il controllo della qualità dell'olio (ad es. l'impiego del microESR per misurare lo stato ossidativo), sulla valorizzazione di sottoprodotti del frantoio (come sansa di oliva, acque reflue, ecc.) come materie prime per diverse applicazioni industriali e sul riutilizzo degli oli alimentari esausti (ad es. oli per friggere) per la produzione di biodiesel.

1. State-of-the-Art

The production of olive oil is of great importance for the high economic, sensory and nutritional value of this food and its relevance in the Mediterranean diet. Olive oil is subjected to oxidation, which, in the case of all the oils and fats, affects the shelf-life due to the appearance of rancid (Diaz-Montana et al., 2023). The oxidation determines the formation of primary and secondary products that can be determined by iodometric titration and spectrophotometric measurement of specific ultraviolet extinctions of conjugated dienes and trienes, respectively. These analytical determinations, reported in Reg. (EU) 2022/2105, are among the quality parameters for establishing the commercial category of virgin olive oils. In fact, an olive oil can be classified as extra virgin if the limits, established by the European Union, relating to free acidity, number of peroxides, specific extinctions in the ultraviolet, organoleptic evaluation (by panel test, in relation to the intensity of the fruity attribute and the eventual presence and intensity of defects) and content in ethyl esters of fatty acids are respected. Innovative, more sustainable analytical methods need to be developed in addition to the official ones, in particular with less use of chemicals and solvents (Valli et al., 2016). In fact, for example, the determination of the number of peroxides by titration has many disadvantages such as long lead times, the amount of sample required, the wide use of solvents and the production of waste (Longobardi et al., 2021). The electron spin resonance represents a possible and promising tool for the evaluation of the oxidation state of olive oil, as it is able to detect the presence of free radicals. In the literature there are studies on the application of electron spin resonance to evaluate oxidative stability in different foods and beverages, such as coffee, wheat flour, bread, peanuts, milk powder, chicken meat (Andersen et al., 2018) and hemp oil (Tura et al., 2019). A very important aspect related with the sustainability of the olive oil supply chain, in addition to post-production quality assessments, is certainly the high amounts of by-products and waste that are generated by the mills. In particular, a large amount of vegetation water is produced, which represents an environmental problem, as their polluting power can cause issues also to soil and groundwater (Gómez-Caravaca et al., 2014). In addition, olive leaves represent another rarely valued waste. A possible scenario sees both these by-products combined and valorized for biogas production. However, the high lignin content in the leaves represents a problem for their conversion into biogas (Romero-Garcia et al., 2014). Given the great importance of developing a sustainable olive oil supply chain and a virtuous oil mill, it is necessary to investigate possible pre-treatments to also make olive leaves an efficient substrate that can be used as raw materials in a biodigester together with vegetation water. Finally, at the end of its life cycle, waste vegetable oils, including cooked olive oils, can be used as substrates for biodiesel production. It is important to investigate their physical and chemical characteristics, such as lipidic content and humidity (Sharma et al., 2021), to assess their ability to be exploited for the biodiesel production. In fact, this valorization would help to avoid dispersion of waste oils in

the environment, contributing to a circular economy and more sustainable vegetable oils supply chain (Azzena et al., 2023).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following future activities (second and third PhD years) according to the Gantt diagram given in Table 1:
A1) **Bibliographical research.**
A2) **Use of microESR to measure olive oil oxidation state**: in A2.1) the first step is to develop a model system to assess microESR values with respect to peroxides, ultraviolet spectrophotometric extinction coefficients, OSI time, fatty acids profile, total phenols, and sensory analysis results. To this aim, an olive oil sample was oxidised with the Rancimat instrument for 3, 6, 12, 15, 18, 21 and 24 h. These amounts of time were chosen because the olive oil that was selected shows an OSI time of 21.7 h and the goal was having 8 points representing the kinetic of the olive oil oxidation. Subsequently, it is planned to construct a statistical model for the estimation of the oxidative state. Afterwards, a study of the oxidative state (A2.2) will be carried out by correlating the microESR values and the results of the abovementioned instrumental and sensory analysis, as well as volatile compounds profiles, on a set of around 100 virgin olive oil samples.
A3) **Valorization of olive mill by-products**: characterization of olive wastewaters, pomace, stones, leaves, and experimental trials on biogas production by using olive wastewaters and leaves as raw materials.
A4) **Characterization of waste vegetable oils for biogas production**: chemical and physical characterisation of waste vegetable oils through the analysis of fatty acids profile, oxidation stability, free acidity, sterols profile, pigments contents, waxes content, and humidity.
A5) **Writing the PhD thesis, scientific papers as well as oral and/or poster presentations.**

Table 2 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Bibliographic research***																								
A2)	***Use of microESR to measure olive oil oxidation state***																								
	1) Set up of the model system: analysis of the olive oil samples																								
	2) Oxidative state study on around 100 olive oils																								
	3) Data analysis																								
A3)	***Valorization of olive mill by-products***																								
	1) Characterization of olive mill by-products																								
	2) Experimental trials on biogas production using wastewaters and olive leaves as raw materials																								
A4)	***Characterization of waste vegetable oils for biogas production***																								
A5)	***Thesis and Paper Preparation***																								

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Investigation of endogenous and/or exogenous phenolic metabolites in humans using *(un)targeted* metabolomics (ENDOPHENOL)

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This PhD research project aims at characterizing low-molecular weight (poly)phenols (LMWP) in biological samples, through metabolomics approaches, in a controlled, (poly)phenol-free diet, with or without coffee, as a known source of LMWP. The main parameters leading to interindividual variability will also be considered. LMWP are common metabolites of endogenous metabolic pathways and dietary proteins, as well as common colonic catabolites of dietary (poly)phenols. However, the contribution of each possible source to the circulating LMWP is poorly understood and a current gap in (poly)phenol research.

Studio dei metaboliti fenolici endogeni e/o esogeni nell'uomo mediante metabolomica *(un)targeted* (ENDOPHENOL)

Questo progetto di tesi di dottorato mira alla caratterizzazione dei (poli)fenoli a basso peso molecolare (LMWP) in campioni biologici, attraverso approcci metabolomici, in un contesto di dieta controllata priva di (poli)fenoli con o senza il consumo di caffè come fonte nota di LMWP. I principali parametri responsabili della variabilità interindividuale verranno presi in considerazione. I LMWP sono metaboliti comuni di alcuni pathway metabolici endogeni, così come possono derivare dal catabolismo colonico dei (poli)fenoli introdotti con la dieta. Tuttavia, il contributo di ciascuna delle possibili fonti di LMWP circolanti è poco conosciuto e rappresenta una lacuna nella ricerca sui (poli)fenoli.

1. State-of-the-Art

Due to their broad spectra of biological activities, plant (poly)phenols are considered important components of the human diet. They represent organic molecules that vary in size and complexity regarding their chemical structure (Vivarelli *et al.*, 2022). After ingestion, the greatest fraction of consumed (poly)phenols follows its path to the large intestine, where they are catabolized by gut microbiota enzymes before entering colonocytes (Rodriguez-Mateos *et al.*, 2014). This means that the route of (poly)phenols from ingestion to bloodstream and urine results in their transformation (75-99%) into a plethora of generally smaller and conjugated catabolites (Scalbert and Williamson, 2000). These metabolites, known as low-molecular weight (poly)phenols (LMWP), appear in plasma in higher concentrations than the parent substances, and are likely responsible for the reported biological activity of plant (poly)phenols.

Among the gaps and challenges in (poly)phenol research, metabolic convergence is a recognized issue in terms of tracking the sources and parent compounds of LMWP derived from the diet (Di Pede *et al.*, 2023). However, one aspect of metabolic convergence is rarely discussed and investigated: the production of certain LMWP species from catecholamines and amino acids and their actual contribution to the pool of bioavailable catabolites. For instance, hippuric acid is an abundant catabolite of the *in vivo* metabolism of many (poly)phenols. However, it is also produced by the glycine deportation system using benzoic acid as means to excrete glycine in urine. Benzoic acid is a common catabolite of numerous (poly)phenol classes, such as flavonoids, but it also derives from phenylalanine/tyrosine metabolism (Vong *et al.*, 2022). Another example, 3′,4′-Dihydroxyphenylacetic acid is originated by the microbial catabolism of several dietary (poly)phenols and is also a catabolite of dopamine (Eisenhofer *et al.*, 2004).

Besides the different possible sources of LMWP, there are factors of variability among individuals, such as age, sex, genetics, and the microbiota diversity, which could influence both concentration and nature of compounds in biological samples (Manach *et al.*, 2005). Accordingly, steps must be taken to understand and consistently report circulating LMWP species, concentrations, and possible sources, to further comprehend their role in nutrition and health, also advancing the field of personalized nutrition.

2. PhD Thesis Objectives and Milestones

During the 1st year of the PhD, the proposed activities were i) literature search and review for the sake of being up to date with the state of the art and ii) conduction of a randomized crossover clinical trial, in which 30 volunteers (adults, aged between 20-40 years old, BMI of 18-28 kg/m^2) followed a personalized and standardized (poly)phenol-free diet

for 5 consecutive days, and, on the morning of the third day, received a single dose of sweetened decaffeinated coffee or sweetened hot water (volume of 180 mL) and samples (urine, blood and faeces) were collected.

The foreseen activities for the 2nd and 3rd years are described below and subdivided according to the Gantt diagram given in Table 1:

A1) **Analyses contemplated within the primary outcome:** characterization of LMWP pool in urine with and without a source of dietary (poly)phenols, to discriminate their endogenous or exogenous origin; identification of potential (poly)phenol catabolites not previously characterized, providing a comprehensive picture of the impact of coffee-(poly)phenol consumption on the urinary metabolome in humans. These analyses will be done by LC-MS targeted and untargeted approach.

A2) **Analyses contemplated within the secondary outcome:** identification of those variables which could be associated with metabolic phenotype or different response to the intervention, specifically for (poly)phenol metabolism. i) Fecal microbiota profiling and characterization to assess the influence of colonic microbiota composition over the endogenous and/or exogenous LMWP production and evaluation of interindividual variability in metabolite production; ii) feces analysis to identify produced but not absorbed exogenous (controlled-diet + coffee) and endogenous (controlled-diet + water) LMWP; iii) genotyping following genome-wide single-nucleotide polymorphism (SNP) analysis.

A3) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Grantt diagram for PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Analyses primary outcome***																								
	1) Targeted metabolomics urine																								
	2) Untargeted metabolomics urine																								
A2)	***Analyses secondary outcome***																								
	1) Microbiota profiling																								
	2) Targeted metabolomics feces																								
	3) Untargeted metabolomics feces																								
	4) Gene SNP analysis																								
A3)	***Thesis and Paper Preparation***																								

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Workshop contributions

2st year - PhD Poster Communications

Low-cost non-destructive sensors for measuring polyphenols and quality attributes in musts and wines

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For the second year of my PhD, I developed a collection of low-cost and non-destructive sensors for monitoring polyphenols fractions in musts and wines. Two prototype spectrophotometers (VIS-NIR) are used to acquire spectra of wines and musts which were then compared with total polyphenol index, anthocyanin, tannin, polymer pigment and colour intensity content, analysed on the same samples, with destructive analytical approaches. Moreover, the concentrations of the different fractions of phenolic compounds in wines have been measured with wet chemistry analysis (HPLC-DAD) and through sensor based on acoustic microwave. Specifically, new chemical functionalization strategies were developed for gold-based acoustic transducers.

Sensori non distruttivi a basso costo per misurare polifenoli e attributi di qualità in mosti e vini.

Nel secondo anno di dottorato ho sviluppato una serie di sensori a basso costo e non distruttivi per il monitoraggio delle frazioni polifenoliche nei mosti e nei vini. Due prototipi di spettrofotometri (VIS-NIR) sono stati utilizzati per acquisire spettri di vini e mosti che sono stati poi confrontati con l'indice di polifenoli totali, il contenuto di antociani, tannini, pigmenti polimerici e l'intensità del colore, analizzati sugli stessi campioni con approcci analitici distruttivi. Inoltre, le concentrazioni delle diverse frazioni di composti fenolici nei vini sono state misurate con analisi di chimica umida (HPLC-DAD) e attraverso sensori basati su microonde acustiche. In particolare, sono state sviluppate nuove strategie di funzionalizzazione chimica per i trasduttori acustici a base di oro.

Key words: Low-cost spectrophotometers, polyphenols, wine, quartz crystal microbalance, VIS-NIR, acoustic microwave.

1. Introduction

In accordance with the PhD project previously described (Alfieri, 2022), this poster reports the main results of the first two-years activities

(A1) Original and polyphenols-enriched wine samples have be analysed through classical analytical approach (HPLC-DAD) to quantify polyphenols content. Quantification has be performed by using calibration curve built with 33 standards. The same samples have been then analysed with the Lab-on-a-Chip biosensor. At this stages, the collected data have been used to perform functionalization tests of quartz microbalances. Two funzionalizations were selected as promising tool to measure polyphenols: Gel-A and MP5 ;

(A2) Spectral acquisitions with VIS and NIR prototype spectrophotometers have been taken on fermenting musts and wines. Spectral measurement were correlated with the total polyphenol index, colour intensity, tannin content, anthocyanin content and poilimer pigment concentration.

2. Materials and Methods

For the first operational objective (A1), the identification, characterization and quantification of the different polyphenols of grapes and wine was carried out by high-performance liquid chromatography (HPLC). The method used for HPLC analysis is reported by Watherouse (1999). Briefly, an HPLC system (Dionex Corporation Sunnyvale, Sunnyvale, CA, USA) with four pumps (P680) of solvent and PDA 100 as detector was used. A C-18 column (Dionex Acclaim® 120 C18, 5 µm, 4.6 × 250 mm), maintained at 40 °C, with a mobile phase flow rate of 0.5 mL/min was used as the stationary phase. The HPLC measurements were then correlated with those obtained with the QMB-D instrument functionalized with i) bovine serum albumin (BSA); ii) type A gelatin for porcine skin (Gel-A); iii) synthetic low-molecular-weight peptide called istatine-5 (Ist-5) and iv) a peptide fragment of the murine salivary protein-5 (MP-5)) (Gagliardi, 2022).

For the operational objective A2, sixteen different micro vinification were conducted with daily sampling until the end of fermentation. On collected samples spectral acquisition were take by using NIR and VIS low-cost prototype spectrophotometers developed by Nature 4.0. On the same samples, wet chemistry analyses were performed (i.e. total polyphenol index, anthocyanin, tannin, polymer pigment and colour intensity as reported by Ribereau-Gayon, 1965; Glories, 1984; Iland, 2000; Mercurio, 2007 respectively). Analytical and nondestructive approaches were then used to built predictive models of the different quality parameters. Moreover, a study of how winemaking condition (i.e. presence of stalks and CO_2) can affect spectral acquisition has also been performed.

3. Results and Discussion

3.1 Measurements with VIS-NIR prototype spectrophotometers

The VIS-NIR spectral acquisition was taken on 160 must/wine samples from 3 different varieties at different degrees of ripeness and on wines made with two different winemaking process. Correlation data between spectra and destructive measurements of the various polyphenols are currently being processed. The figure 1 shows some results of the spectra of the VIS prototype.

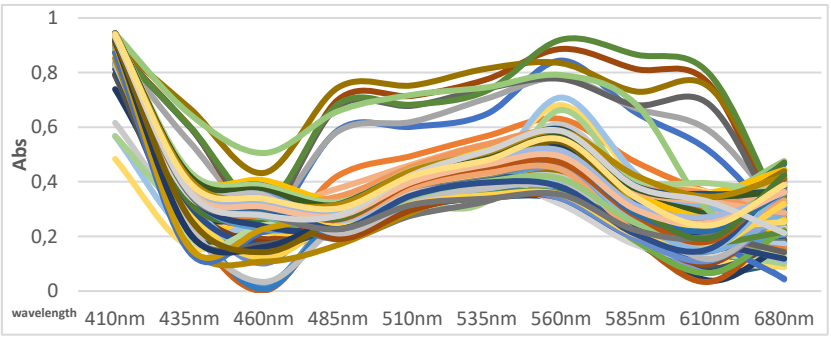

Figure 1. *Spectral trends of the 160 must/wine samples, the VIS prototype covers 8-point wavelengths between 410nm to 860nm.*

3.2 Modelling of the QCM-D sensor

For measurements with gravimetric sensors based on acoustic wave QCM-D, monitoring data of changes in resonance frequency, Δf, are used as units of measurement, allowing small changes in crystal thickness (mass) to be detected. The dissipation, ΔD, gives information about the energy losses in the system and are particularly useful in the study of soft layers, where this information is used for quantification of the layer properties. Via an applied voltage, the crystal can be excited to resonance, and the resonance frequency is related to the thickness (mass) of the disk. Particularly, it has been observed that microbalances functionalised with MP5 and Gel-A effectively detect polyphenols in commercial wines. In particular, MP5 can predict polyphenols content (with an R2 of 0.759 – data not shown) as well as different classes of polyphenols such as the level of hydroxybenzoic acids (figure 2).

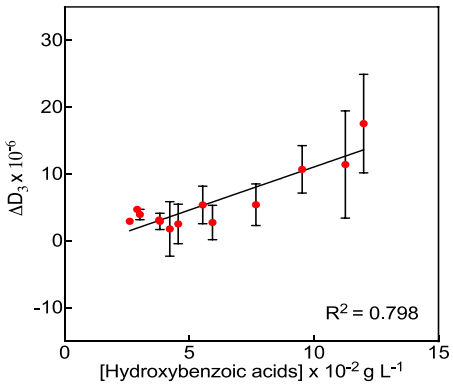

Figure 2. *Correlation model for hydroxybenzoic acids using the ΔD of QCM-D functionalised with MAP-5*

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Development of a multifunctional cooking appliance: evaluation of food quality indexes and cooking functions

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Two activities related to the identification of quality indexes, in representative foods, for high temperature cooking functions are presented. The first activity aims to identify an objective method for measuring crispness that can be correlated to the sensory perception of this attribute in products cooked by air frying. The second activity concerns identifying and evaluating qualitative indexes linked to browning and thermal damage, cooked at high temperatures in domestic appliances.

Sviluppo di un apparecchio di cottura multifunzionale: valutazione di indici di qualità degli alimenti e di programmi di cottura

Vengono presentate due attività legate all'individuazione di indici di qualità per funzioni di cottura ad alta temperatura. La prima attività ha lo scopo di individuare un metodo oggettivo per la misura della croccantezza correlabile alla percezione sensoriale di questo attributo in un prodotto modello sottoposto a frittura ad aria. La seconda attività riguarda l'individuazione e la valutazione di indici qualitativi, legati all'imbrunimento e al danno termico, della pizza nella cottura a temperature elevate in forni domestici.

Key words: high temperature cooking, thermal damage, color, mechanical-acoustic properties

1. Introduction

In accordance with the PhD thesis project, as presented in Aliberti (2022), this report regards 1) the development of a method able to discriminate crispness degree of an air fried food through the correlation between mechanical-acoustic parameters and sensory perception; 2) the identification of analytical indexes to evaluate and compare quality characteristics of pizza (i.e., weight loss, color development and thermal damage), baked using high temperature conditions (>300°C) in two home-cooking appliances, both compared to the conventional baking.

2. Materials and Methods

2.1 Crispness evaluation

Frozen chicken nuggets were used as a model food and air fried (Philips Airfryer XXL) using different time-temperature conditions (from 180°C to 200°C for 10 to 20 minutes). Mechanical-acoustic tests were carried out on a TA.XTplus Texture Analyzer (Stable Micro Systems, Surrey, U.K.) coupled with an Acoustic Envelope Detector (Stable Micro Systems, Surrey, U.K.). Energy, Number of Force Peaks (NFP), Force at 1st Peak and at 2nd Peak were obtained from the cutting test response (Varela et al., 2008). Maximum Sound Pressure Level (Smax), N° of Sound Peaks (NSP), Linear Distance of Sound (LDS), were captured modifying the method described by Varela et al. (2008). Cooking process indexes (weight loss, WL; whole and crust moisture, Mw and Mc) were also evaluated. A ranking test was used for sensorial evaluation of crispness: 24 trained assessors were asked to rank five samples from lowest to highest crispy. Data from mechanical-acoustic tests were analyzed by one-way analysis of variance (ANOVA), followed by Fisher's LSD test to highlight significant differences ($P< 0.05$) among samples, by STATGRAPH plus 5.1 (Statistical Graphics Corp., Herndon, VA, USA). Ranking data (as the sum of induvial ranks, $\sum$ ranks) were analyzed by Friedman analysis of variance to verify the existence of significant preference differences among the samples (Lawless & Heymann, 2010). The Fisher's Least Significant Difference was used to identify the samples that differed among themselves at 5% of significance.

2.2 High temperature pizza cooking

Homemade pizzas were prepared following a traditional recipe. The cooking performance was followed at different high temperature conditions. Three ovens were used: an electric high temperature pizza oven (P134H, EffeUno Srl, Limena, PD, Italy) working at 450°C (HiT), a commercial oven (BIM19700DXMS, Beko Srl-Arçelik AŞ, Istanbul, Turkey) with an automatic pizza function, working at 310°C (MeT), and a conventional baking oven (AKZ9 6270 IX, Whirlpool Corporation, Mi, USA) working at 250°C (LoT). Each appliance was preheated before the baking tests. WL% was evaluated gravimetrically; Browning Index, BI and Intensity Mean, IM were evaluated on pizza crust by image analysis software Image-Pro® v10 (Media Cybernetics, Rockville, MD, USA) to assess color development. The content of 5-hydroxymethylfurfural, HMF (determined by HPLC) and the Maillard Reaction Products, MRPs (determined spectrophotometrically) of pizza crust were considered as thermal damage indexes (Giovanelli & Cappa, 2021). Data were analyzed by one-way analysis of variance (ANOVA), followed by Fisher's LSD test to highlight significant differences ($P < 0.05$) among samples at the final cooking time.

3. Results and Discussion

3.1 Crispness evaluation

Table 1 reports the data obtained by the mechanical-acoustic tests on chicken nuggets air fried at different t/T conditions, together with the sensory test results. Data trends and statistical significance are similar for Energy, NSP, LDS and ∑ ranks. Crispness increases from least crispy nuggets (180°C x 10min) to most crispy nuggets, obtained at 190°C x 20min. Coherently, WL% increase while Mw% and Mc% decrease. Among mechanical properties, energy is directly proportional to WL% (R^2= 0.947) while regarding acoustic properties, NSP and LDS are inversely correlated to Mw% (R^2= 0.976 and 0.960, respectively). Sensory data are statistically correlated to Energy (R^2= 0.950), NSP (R^2= 0.965) and LDS (R^2= 0.981). The correlation between sensorial perception of crispness and textural and acoustic characteristics is verified and will allow to estimate sensorial crispness by an instrumental objective evaluation method.

Table 1 Results of instrumental and sensory tests of air fried chicken nuggets samples at different time-temperature conditions.

Sample	Mechanical test				Acoustic test			Sensory test
	Energy (10^{-3}N·m)	NFP	Force 1st peak (N)	Force 2nd peak (N)	Smax (dB)	NSP	LDS (10^{-3} dB·m)	Σ ranks
180°Cx10'	218±30^{a}	3.7±0.8^{a}	16.0±1.5^{a}	15.0±1.7^{a}	71.8±5.0^{a}	53.1±17.1^{a}	6481±714^{a}	24^{a}
180°Cx15	299±33^{b}	3.7±1.2^{a}	21.4±3.0^{b}	25.5±6.7^{b}	81.0±2.7^{b}	120.9±38.4^{b}	10400±2265^{b}	53^{b}
190°Cx15	325±41^{bc}	5.1±1.8^{ab}	23.1±2.0^{b}	29.3±6.9^{b}	82.1±2.9^{bc}	186.7±53.3^{c}	14543±3029^{c}	74^{bc}
190°Cx20	442±54^{d}	10.7±2.4^{c}	30.4±4.2^{c}	40.6±10.9^{c}	84.1±1.3^{c}	260.9±40.3^{d}	18979±2434^{d}	117^{d}
200°Cx15	342±61^{c}	6.8±2.7^{b}	23.1±2.4^{b}	32.4±11.6^{b}	81.7±3.0^{bc}	184.6±45.6^{c}	15211±2893^{c}	92^{c}

3.2 High temperature pizza cooking

Cooking tests show similar final WL for HiT and MeT and evidence that thermal damage indexes increase at all baking temperatures. HFM level is similar in pizza baked at MeT and LoT; at HiT, HMF level increases sharply after 2 min cooking time. The increase in MRPs with cooking time is similar to HMF formation. Considering the optimal cooking time at each temperature, HiT and MeT pizza reached a similar percentage WL (9.1-10.6%), lower than that obtained at LoT (15%). HiT cooking resulted in the highest levels of HMF and MRPs; similar and definitely lower values were detected in LoT and MeT cooking. Concerning color development, the samples differed significantly: both IM and BI indexes showed the lowest and highest values in HiT, which appeared darker, while pizza crust baked at MeT was slightly browner than the pizza crust baked at LoT.

Table 2 Cooking parameters of pizzas cooked at different time-temperature conditions.

Sample	T (°C)	t (min)	WL (%)	HMF (g/kg dw)	AU 280 (AU/kg dw)	AU 360 (AU/kg dw)	AU 420 (AU/kg dw)	IM	BI
HiT	450	2.5	9.1±1.0^{a}	37.6±3.0^{b}	1047.0±126.0^{b}	202.3±21.1^{b}	80.9±9.8^{c}	101.0±13.0^{a}	54.6±6.2^{b}
MeT	310	8	10.6±0.1^{a}	2.5±0.2^{a}	675.5±4.0^{a}	60.3±1.2^{a}	14.8±1.2^{a}	164.9±20.2^{b}	29.2±6.3^{a}
LoT	250	14	15.0±1.2^{b}	2.0±0.6^{a}	765.4±80.0^{a}	118.2±2.9^{b}	64.4±2.4^{b}	176.4±7.2^{b}	24.6±2.3^{a}

These data indicate that very high baking temperatures can produce significant thermal damage, potentially harmful for health.

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Mitigation of environmental impacts caused by farm chemicals and proposal of new certification procedures

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The spraying of pesticides has been a crucial step in the food system while distributing pesticide droplets precisely to a target area has always been a challenge. To solve this problem the AgroForestry Innovations Lab at the Free University of Bolzano is researching in collaboration with local farmers and machine builders to enhance the precision of pesticide droplet distribution in South Tyrol, Italy. In the first part we did Particle/Droplet Image Analysis (PDIA) and an Oxford Lasers N60 shadowgraphy to collect data for nozzles characterization. We investigated a simplified deposition assessment strategy involving Uranine tracer. This data will aid in evaluating nozzle performance and developing models to predict drift during chemical treatments.

Mitigazione degli impatti ambientali causati da farm chemicals e proposta di nuove procedure di certificazione

L'irrorazione di pesticidi è stata una fase cruciale nel sistema alimentare, mentre la distribuzione di goccioline di pesticidi in modo preciso su un'area target è sempre stata una sfida. Per risolvere questo problema, l'AgroForestry Innovations Lab della Libera Università di Bolzano sta conducendo ricerche in collaborazione con agricoltori locali e costruttori di macchine per migliorare la precisione della distribuzione delle goccioline di pesticidi in Alto Adige, Italia. Nella prima parte abbiamo eseguito Particle/Droplet Image Analysis (PDIA) e un'ombreggiatura Oxford Lasers N60 per raccogliere dati per la caratterizzazione degli ugelli. Abbiamo studiato una strategia di valutazione della deposizione semplificata che coinvolge il tracciante dell'uranina. Questi dati aiuteranno nella valutazione delle prestazioni degli ugelli e nello sviluppo di modelli per prevedere la deriva durante i trattamenti chimici.

Key words: Data acquisition, Drift and spraying,Nozzles, Image Analysis, MATLAB.

1. Introduction

In accordance with the PhD thesis project, this poster reports the main results of the first two activities concerning:
(A1) The characterization of individual nozzles via VisiSizer N60V Laser System
(A2) Evaluation of different tracers for quick and cost-effective spray deposition measurements

2. Materials and Methods

A typical Nozzle examination with N60 for test methodology development at two pressures each: Three standard hollow-cone nozzles and their anti-drift (air-inclusion) equivalent were evaluated at 4 and 8 bar pressure. Before proceeding with the test, the flow rate of each nozzle is tested with the aid of a dedicated device provided by composed of a tight-fitting tube collecting the nozzle output and conveying it to a container. The pump was run for a time of at least 90 seconds, after which the collected liquid is weighted to an accuracy of ±1 g and divided by the elapsed time to obtain the flow rate. The nozzle orifice is placed 30 cm above the center of the laser FOV. The procedure is as follows. After mounting each nozzle on the holder, the circuit is primed with the pump set to the first desired pressure with an accuracy of ±0.1 bar. An image acquisition sequence (Kashdan et al., 2003, Kashdan et al., 2007) is commanded in each of three different positions of the nozzle concerning the laser FOV making sure to acquire at least 10000 droplets per acquisition. Evaluation of different tracers for quick and cost-effective spray deposition measurements (Grella et al., 2021.) Smart fertilizers/pesticides spraying technology for precision agriculture and the environment we investigated a simplified deposition assessment strategy involving uranine, a non-toxic and low-cost fluorescent tracer widely used in other fields to minimize the measurement uncertainties exploiting the well-known phenomenon of optical absorbance. A nozzle evaluation bench has been used to deposit the fluorescent solution on a matrix of Petri dishes, which were then oven-dried, and the residuals redissolved in a fixed amount of water. Spectrophotometry was used to retrieve the mass of the deposited solution. After careful calibration against known uranine concentrations, the method yielded results very well correlated to the weight measurements performed before drying and allowed us to trace back an approximate deposition curve. The complete evaporation of the deposited solvent gets rid of the unpredictable atmospheric conditions during the test, while the flexibility of the solution allows one to easily tailor the technique to different application volumes, deposition rates, or collector configurations without losing accuracy. After oven-drying the samples for 18h at 60°C, the residual material was dissolved in 25ml of DI water and the initially deposited material has been estimated from the absorption spectrum (Becce, 2022).

3. Results and Discussion

3.1 Determination of the main physical properties

An example of droplet spectra for the same nozzle operated at different pressures is presented in figure 1, both in terms of drop size distributions (DSD, histogram) and cumulative distribution functions (CDF, solid lines). When compared to the drop spectrum of an equivalent, conventional nozzle, the working principle of the air induction nozzle becomes evident.

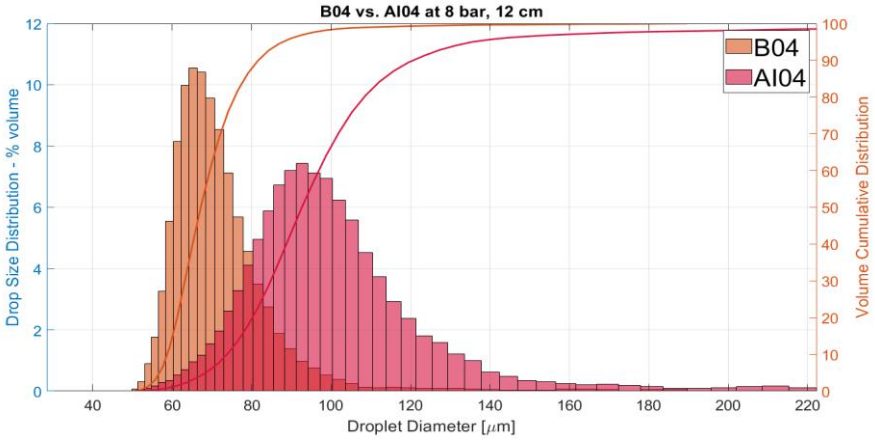

Fig. 1: The B04 vs AI01 nozzle at 4 and 8 bar pressure.

The figure shows the much broader, coarser droplets produced by AI04 with respect to B04. The decrease in size of the droplets is evident with the greater pressure for the AI0.

3.2 Evaluation of Fluorescence tracers for cost-effective spray deposition measurements

The calibration curve has been calculated by depositing a known amount of the sprayed solution and adding DI water to reach the fixed volume used for redissolving the material in the other container.
The negative values for the estimated curve around zero are due to the intercept in the calibration equation. Indicating a threshold of deposited material, from this estimation is possible to identify the minimum distance beyond which less than a certain amount of solution has been deposited.

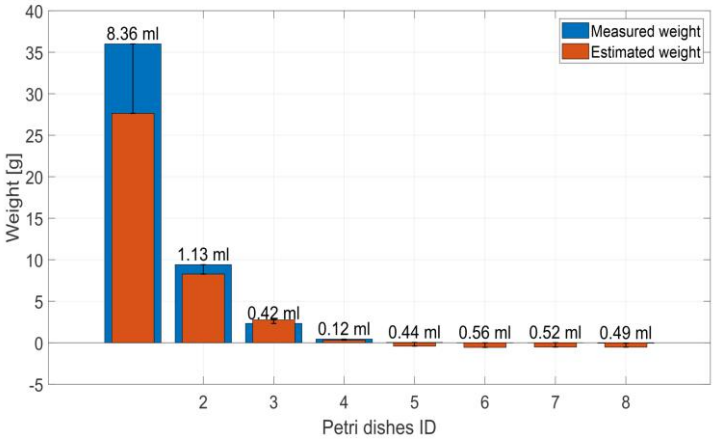

Fig. 2: The calibration curve of Uranine representing estimated weight vs measured weight.

The present work focused on developing a preliminary nozzle test methodology and acquire the know-how necessary to assess nozzle performances. A data parsing system was set up in MATLAB to gather the necessary relevant parameters with routines available to plot several parameters and compare them between tests, while keeping a high level of flexibility. To create an in-house reference database, the next step is to standardize the analysis and apply it to the rest of the stock available at the AFI-Lab.

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Study of the relationship between gluten-free foods and microbiota in celiac subjects

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The activities carried out in the second year of PhD project are below described. The present work is aimed at characterizing a gluten-free laboratory bread (B) using rice flour supplemented with an artichoke leaf powder extract (AE). The AE has been added to the gluten-free experimental bread. Four different batches of bread were prepared: two mixtures (SB and SB-AE) with the addition of a gluten-free type II mother yeast (tII-SD) and two control mixtures (YB and YB-AE) not containing tII-SD. The purpose of this study is to modify a traditional food product consumed daily worldwide to support celiac patients.

Studio della relazione tra alimenti gluten-free e microbiota dei soggetti celiaci

Le attività svolte nel secondo anno di dottorato sono descritte. Il presente lavoro mira a caratterizzare un pane da laboratorio senza glutine (B) utilizzando farina di riso integrata con un estratto di foglie di carciofo in polvere (AE). L'AE è stato aggiunto al pane sperimentale senza glutine. Sono stati preparati quattro diversi batch di pane: due miscele (SB e SB-AE) con l'aggiunta di un lievito madre di tipo II senza glutine (tII-SD) e due miscele di controllo (YB e YB-AE) senza tII-SD. Lo scopo dello studio è modificare un alimento tradizionale consumato quotidianamente nel mondo per supportare i pazienti celiaci.

Key words: *celiac disease, artichoke, antioxidant, nutrigenomics.*

1. Introduction

Gluten-free products have gained significant attention due to the increasing prevalence of gluten-related disorders and the growing consumer demand for gluten-free alternatives. Artichoke (Cynara scolymus) leaf extract has been reported to possess various bioactive compounds with potential health benefits, including anti-inflammatory properties. This study aimed to evaluate the nutritional profile, impact on gut microbiota, and anti-inflammatory response of a gluten-free bread formulation enriched with artichoke leaf powder extract.

2. Materials and Methods

In the study, Type-II sourdough (tII-SD) with a dough yield (DY) of 200 was prepared using commercial rice flour and a single-strain inoculum of Leuconostoc pseudomesenteroides DSM 20193. Six batches of tII-SDs were prepared by varying incubation temperatures (20, 25, and 30 °C) and starter cell densities (6 or 7 log CFU/mL). Gluten-free bread batches were manufactured at the pilot plant of the Department of Soil, Plant, and Food Science of the University of Bari. Four batches of bread were used in the study: (i) baker's yeast gluten-free bread without sourdough and artichoke extract (YB), (ii) baker's yeast gluten-free bread with artichoke extract (YB-AE), (iii) tII-SD gluten-free bread (SB), and (iv) tII-SD gluten-free bread with artichoke extract (SB-AE). An artichoke leaf powder extract characterized by 5% titratable chlorogenic acid stabilized in maltodextrins was provided by Farmalabor S.r.l. To simulate *in vitro* digestion, standardized procedures involving oral, gastric, and intestinal phases were followed. The bread samples underwent simulated in vitro digestion, and during this process, an aliquot of fermented fecal batch was used to analyze the volatile organic compound (VOC) profiles. The VOC analysis was performed using solid-phase microextraction (SPME) with a divinylbenzene/Carboxen/polydimethylsiloxane fiber, and the volatile compounds were analyzed using gas chromatography. Two different human cell lines, Caco-2 ICLC HTL97023 and human keratinocyte NCTC 2544, were used in the experiments. These cell lines were obtained from the National Institute for Cancer Research of Genoa. The anti-inflammatory properties of the digested bread samples were assessed by measuring the expression levels of tumor necrosis factor-alpha (TNF-α) and interleukin 1-β in Caco-2 cells.

3. Results and Discussion

3.1. Fecal Volatile Organic Compounds in Fecal Batches

The study conducted metabolic profile characterization of fecal batches after 20 hours and 42 hours of incubation

using qualitative and quantitative analysis of volatile organic compounds (VOCs). A total of 59 volatile metabolites were identified and classified into different chemical classes, including alcohols, aldehydes, esters, hydrocarbons, indoles, ketones, organic acids, phenols, and terpenes. Additionally, four compounds that did not belong to these classes were also identified. The presence of specific metabolites with potential health benefits was evaluated in the residual from fecal microbiota fermentation. After 20 hours of fermentation, the profiles of VOCs distinguished the tII-SD gluten-free bread (SB) samples from the baker's yeast gluten-free bread (YB) samples. Aldehydes and hydrocarbons were more prevalent in the YB samples, while their presence was not significantly influenced by the addition of artichoke extract (AE). However, high scores of hydrocinnamic acid were specifically observed in SB-AE-T20. After an additional 22 hours of incubation (42 hours in total), a wider range of organic acids, phenols, and indoles were detected in the samples containing artichoke extract (SB-AE). Statistically significative differences in the above-mentioned compounds were found as a consequence of the artichoke extract addition. Interestingly, although the artichoke extract was also added to the baker's yeast gluten-free bread (YB-AE) samples, hydrocinnamic acid and cyclohexanecarboxylic acid were not observed to the same extent as in SB-AE. This led us to hypothesize a positive interaction between the microbiota of tII-SD and the metabolism of artichoke extract. These differences in the metabolic profiles of the samples may explain the variations in anti-inflammatory activity observed when Caco-2 cells were exposed to lipopolysaccharide (LPS). The presence of specific metabolites, such as hydrocinnamic acid, in the SB-AE samples could potentially contribute to enhanced anti-inflammatory effects.

3.2. Anti-Inflammatory Effects

The results of the study indicate that the supernatants from colonic fermented bread samples, specifically SB-AE (T20 and T42), exhibited significant anti-inflammatory activity in Caco-2 cells. The expressions of pro-inflammatory cytokines, TNF-α and IL-1β, were measured to assess the anti-inflammatory effectiveness of the supernatants. When compared to the positive control (LPS from E. coli), SB-T42, SB-AE-T20, and SB-AE-T42 demonstrated a significant decrease in TNF-α expression. Notably, SB-AE-T42 showed no significant difference from the negative control (cells not exposed to LPS), indicating its potential in suppressing TNF-α production even in the presence of a pro-inflammatory trigger. In terms of IL-1β expression, both supernatants from SB-AE (T20 and T42) exhibited a significant reduction compared to the positive control. Furthermore, these two supernatants did not significantly differ from the negative control, suggesting their ability to effectively inhibit IL-1β expression even in the presence of LPS. Overall, the highest anti-inflammatory effectiveness was observed in the supernatants from SB-AE samples. These samples exhibited a significant reduction in both TNF-α and IL-1β expressions under all tested conditions in Caco-2 cells exposed to LPS. This indicates that the colonic fermentation of bread, along with the incorporation of artichoke leaf powder extract (AE), enhanced the anti-inflammatory properties of the bread samples. These findings highlight the potential of colonic fermented bread supplemented with artichoke leaf powder extract in reducing pro-inflammatory cytokine expressions, specifically TNF-α and IL-1β, which are associated with inflammatory processes.

4. Conclusion

Based on these results, the study supports the application of artichoke leaf extract as a functional ingredient in the development of gluten-free products with improved biological properties. The use of artichoke extract in the formulation of gluten-free products could potentially contribute to reducing inflammation and oxidative stress in individuals with celiac disease. Furthermore, the study suggests that research focused on artichoke extract could pave the way for innovative and customized therapies in the nutritional management of celiac disease. The data collected from this research can be integrated into the broader field of research exploring the combination of various dietary components to design functional and clean label products. Overall, the study highlights the potential of artichoke leaf extract as a dietary supplement for individuals with celiac disease, providing evidence of its positive effects on oxidative stress and proinflammatory cytokine expression. Further research is warranted to elucidate the underlying mechanisms and evaluate the impact of these bread samples on inflammatory disorders in vivo.

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Impact of vine shoots xylooligosaccharides on the nutritional, technological, and sensory properties of spreadable goat cheese

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A spreadable goat milk cheese fortified with a rich-xylooligosaccharides extract (XE) was developed. The experimental samples were analysed for proximate composition, colorimetric and textural properties. Finally, the sensory features were evaluated.

Impatto di xilooligosaccaridi estratti da tralci di vite sulle proprietà nutrizionali, tecnologiche e sensoriali di formaggio spalmabile di capra

Le attività del progetto di tesi di dottorato hanno previsto la produzione di formaggio spalmabile di capra fortificato con estratto ricco in xilooligosaccaridi (XOS). Successivamente i campioni ottenuti sono stati caratterizzati valutando la composizione centesimale, il colore e le caratteristiche strutturali. È stata inoltre effettuata la valutazione del profilo sensoriale.

Keywords: spreadable goat cheese; vine shoot; xylooligosaccharide extract.

1. Introduction

This poster reports the main results of the activities concerning the manufacturing and characterization of a spreadable goat cheese fortified with XE obtained from vine shoots by steam explosion.

2. Materials and Methods

2.1.Preparation of the XE and spreadable goat cheese production

The XE was extracted from vine shoots with the steam explosion technique, operating at 210 °C for 5 minutes. XOS concentration in the extract was 34 g/L. The XOS syrup was freeze-dried and stored at -20 °C. Raw goat milk was pasteurized at 65 °C for 30 minutes in a thermostatic bath. After lowering the temperature to 30 °C, a mixed starter culture (*Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus*) and a small amount of rennet were added (75 µL/L). Coagulation occurred at 30 °C overnight, then the curd obtained was cut into fragments of about 3 centimetres to allow a slight whey draining. The curd obtained was added with 5.5 g of XE per 100 g of product and homogenized, after that the cheese was placed in hermetically sealed jar, pasteurized at 65 °C for 30 minutes, and stored at 4 °C. A cheese sample prepared without XE was used as control (FC).

2.2. Characterization of FC and FX

Protein, ash, lipid, and total fiber content were determined using the AOAC methods 979.09, 923.03, 945.38, and 991.43, respectively. Lactose, galactose, and organic acids were determined by high-performance liquid chromatography (HPLC) (Trani et al., 2017; Buffa et al., 2004). Moisture was determined by a moisture analyser MAC 110/NP. The pH was measured with a pH-meter. Water activity (a_w) was determined by using the water activity meter Aqua Lab 4TE. The CM-600d colorimeter (Konica Minolta, Tokyo, Japan) and SpectraMagic NX software were used for the color analysis. Brightness (L^*), red index (a^*), and yellow index (b^*) are considered in accordance with the International Commission on Illumination. Back extrusion was evaluated according to De Angelis et al. (2022). Sensory analysis of cheeses was performed by a semi-trained sensory panel composed of ten members. The descriptors were selected considering the ONAF (Italian Organization of Cheese Tasters) vocabulary (Gambera, 2008) were rated on a 1-5 score range. Minitab19 (Minitab Inc., State College, PA, USA) was used for the statistical analysis of all results, reported as mean ± standard deviation (SD) of three replications. To evaluate the differences between samples, one-way ANOVA followed by Tukey's HSD test was applied.

3. Results and Discussion

The addition of XOS caused a slight increase in pH, as the pH value of the extract was 4.40. The addition of XE in FX led to about 5% decrease in moisture, probably due to a higher syneresis during the homogenization process. In addition, non-digestible oligosaccharides such as XOS contribute to an increase in soluble solids in cheese formulation with a consequence on product moisture. No significant differences were found for water activity, lipid, lactose, galactose, and organic acids. An opposite trend occurred for proteins that were found to be greater in FX. The addition of plant extracts may promote the fixation of soluble proteins to the para-casein network or the formation of small protein aggregates, resulting in an increase in the protein content of cheeses (da Silva et al.,

2015). The results of the total dietary fibers highlight the high concentration of fibers in FX. One of the objectives of this study was the enrichment of spreadable goat cheese with xylooligosaccharides for the attribution of the claim "*Source of fiber*" (at least 3 g of fiber per 100 g of product in accordance with EC Regulation No. 1924/2006). The results show that the amount of fiber found in functional cheese was 3.56 g/100g of cheese.

Table 1. Proximate composition (g/100g) of FC and FX.

	Moisture	a_w	pH	Lipid	Total fiber	Protein	Lactose	Galactose	Lactic acid	Acetic acid	Ash
FC	74.6± 0.84^{A}	0.99± 0.00^{A}	4.12± 0.01^{B}	11.89± 0.26^{A}	nd	8.88± 0.04^{B}	1.63± 0.18^{A}	0.60± 0.12^{A}	0.67± 0.27^{A}	0.31± 0.14^{A}	0.63± 0.03^{B}
FX	71.17± 0.91^{B}	0.99± 0.00^{A}	4.22± 0.01^{A}	11.40± 0.10^{A}	3.56± 0.09	9.42± 0.10^{A}	1.60± 0.20^{A}	0.56± 0.06^{A}	0.61± 0.10^{A}	0.35± 0.17^{A}	0.99± 0.05^{A}

FC, spreadable goat cheese; FX, spreadable goat cheese with xylooligosaccharides extract; a_w, water activity.
Results are expressed as mean±standard deviation. Analysis performed in triplicate. Different letters indicate statistical differences according to Tukey's test ($p < 0.05$).

Table 2. Colorimetric and textural properties of FC and FX.

	L*	a*	b*	Firmness	Consistency	Cohesiveness	Viscosity
FC	82.17± 0.04^{A}	-1.51±0.00^{B}	10.47±0.06^{B}	2.38±0.30^{A}	19.54±1.09^{A}	-0.87±0.10^{A}	8.54±0.91^{A}
FX	50.78± 0.44^{B}	7.93±0.22^{A}	26.11±0.51^{A}	1.74±0.07^{B}	12.34±1.06^{B}	-0.66±0.22^{A}	5.53±0.63^{B}

FC, spreadable goat cheese; FX, spreadable goat cheese with xylooligosaccharides extract; L*, brightness; a*, red index; b*, yellow index.
Results are expressed as mean±standard deviation. Analysis performed in triplicate. Different letters indicate statistical differences according to Tukey's test ($p < 0.05$).

Figure 1. Sensory analysis of FC and FX. *, statistically different values ($p < 0.05$).

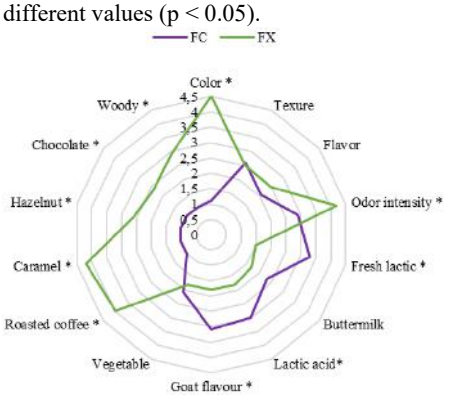
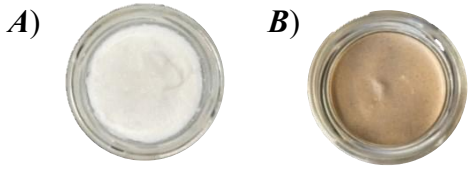


Figure 2. Appearance of FC (***A***) and FX (***B***).



Concerning color analysis, the addition of XE determined a decrease in *L** for FX and an increase of *a** and *b** values. FX showed a browning effect (***Figure 2B***), caused by dark pigments in XE. In general, the addition of by-product extracts or flours in the cheese formulation leads to a tendency to decrease the brightness in the final product and to increase the red and yellow parameters depending on the type of extract added (Difonzo et al., 2023). Significant differences between the two samples were also found for back extrusion analysis. In FX was found a decrease in firmness, related to an increase in pH, as mentioned above. The homogenization process for incorporating XE in the cheese may be the cause of a reduction in cheese consistency. This phase of the production process subjected the cheese to greater mechanical stress that was reflected in the consistency parameter. The addition of XOS extract also caused a decrease in cheese viscosity, and an increase in cohesiveness. Textural features may depend on the particle size of the extract and the interaction between the insoluble fibres and the protein gel created during cheesemaking (Xue et al., 2020). The addition of XE significantly affected the cheese's sensory features (***Figure 1***). The results of the visual analysis confirmed the instrumental color analysis. Maillard compounds present in XE thus influenced the final color of FX. The flavor, which includes sweet, salty, acid, bitter, and astringent was similar in FC and FX. Thus, it can be stated that the addition of XE resulted in a limited change in the perception of basic tastes. The same trend was found for the texture attributes. The taste-olfactory attributes showed strong differences between the two samples. The odor intensity increased significantly in FX. No significant differences were found for vegetable and buttermilk descriptors. The significantly higher attributes in FX were roasted coffee and caramel. The hazelnut, chocolate, and woody odors showed a significant increase in FX and were absent in CT. The overall acceptability was not significantly different (3.6 FC and 3.5 FX). In general, the experimental cheese showed a greater complexity of smells and flavors that was appreciated by the group of tasters.

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E-nose analysis to detect the milk of ruminants fed with the inclusion of fresh forage

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Electronic nose (E-nose) analysis was advantageously used to authenticate milk from highly biodiverse pasture-fed goats and to authenticate milk from buffaloes fed fresh hydroponic barley forage. SPME-GC/MS analysis was conducted to explore the volatile organic compounds (VOCs) that contributed to discriminate the odour of the two types of milk according to the diet fed to ruminants.

Analisi E-nose per autenticare il latte di ruminanti alimentati con foraggio fresco

L'analisi E-nose è stata utilizzata per autenticare il latte di capre alimentate al pascolo ad alta biodiversità e per autenticare il latte di bufale alimentate con foraggio fresco d'orzo idroponico. Poi, è stata condotta un'analisi SPME-GC/MS per identificare e quantificare i composti volatili che hanno contribuito a discriminare l'odore delle due tipologie di latte, in accordo con la dieta somministrata ai ruminanti.

Key words: Pattern recognition, raw milk odour, animal diet, aroma compounds, rapid control analysis.

1. Introduction

In accordance with the PhD thesis project previously described (Balivo, 2022), for the two experimentations on small (A) and large ruminants (B), this poster reports the main results concerning:

(1) the classification of raw goat (A) and buffalo (B) milk according to the animal diet, performed by electronic nose (E-nose) analysis;
(2) the identification and quantification of VOCs to explain the differences in the response of the E-nose sensors to the different types of milk samples, carried out by SPME-GC/MS analysis.

2. Materials and Methods

Milk samples were taken from a total of 90 Saanen goats (divided into two groups, stall group and pasture group, named S and P respectively) and 108 Italian Mediterranean buffaloes (divided into three groups, control group fed with maize silage as forage source, and two experimental treatments in which maize silage was replaced by hydroponic barley forage at 50% and 100%, named C, LH and HH respectively). Concentrate:forage ratio was constant (70:30 and 60:40 for A and B respectively). Goat milk samples were collected in 5 different milking days, while buffalo milk samples in 3 different milking days. Further sampling information has been previously described (Balivo, 2022).

For the analytical methodology, a detailed description is provided in Balivo et al. (2023). Briefly, 2 mL of sample was transferred into 20 mL glass vial with a Teflon/silicon septum in the screw cap and incubated at 25 °C for 30 min to analyse the odour fraction by E-nose. A portable E-nose PEN2 (Airsense Analytics GmbH, Schwerin, Germany), operating with 10 Metal Oxide Semiconductor sensors, was used at constant velocity (400 mL/min). The mean G/G0 values of each sensor response were calculated from measurements in the 55–59 s range (stability of the sensors) using Winmuster v.1.6 software (Airsense Analytics GmbH, Schwerin, Germany).

VOCs were extracted by adding 22.5 g of milk, 20 µL of 2-methyl-3-heptanone as internal standard (389 mg/L) and 2.75 g of sodium phosphate monobasic to a 50 mL glass bottle. The sample was magnetically stirred for 5 min at 55 °C. SPME 2 cm fibre (50/30 µm thick DVB/CAR/PDMS) was inserted through the Teflon septum in the bottle and exposed to headspace for 60 min at 55 °C while stirring. GC was equipped with a Zebron ZB-WAX capillary column (60 m×0.25 mm i.d.×0.25 µm film thickness; Phenomenex, Torrance, CA, USA). The carrier gas was helium with a flow of 1 mL/min. The temperature program was 40 °C for 10 min, then raised at 5 °C/min to 240 °C and held for 11 min. Identification was performed by comparison with pure reference compounds and use of NIST database. Quantification was carried out by normalising the peak areas of each compound with respect to the area of the internal standard peak (MSD ChemStation 5975 TAD Data Analysis software).

3. Results and Discussion

3.1 Electronic Nose Analysis

Figure 1 shows the linear discriminant analysis (LDA) plot obtained from the data vectors extrapolated from E-nose measurements, for goat (A) and buffalo (B) milk samples. E-nose provided good discrimination of milk samples in both investigations (A and B), according to animal diet.

Table 1 shows the confusion matrix with classification of milk samples obtained by LDA analysis. The correct classification of goat milk samples according to pasture and stall diet was 88%. P milk was misclassified as S milk seven times, while S milk was misclassified only three times as P milk.

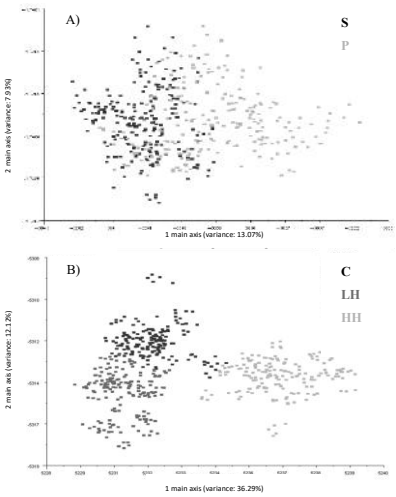

Figure 1 *LDA plot of E-nose data pattern extrapolated in the time range 55-59s (stability of sensors) from 45 Pasture (P) and 45 Stall (S) goat milk (A) and from 108 milk samples obtained from maize silage-fed buffaloes (C) and hydroponic forage-fed buffaloes, with 50% (LH) and 100% (HH) silage replacement percentage (B). Data processed with Winmuster v.1.6 software (Airsense Analytics GmbH, Schwerin, Germany).*

Table 1 *Confusion matrix of the Pasture (P) and Stall (S) goat milk samples (A), and of the buffalo milk samples (B) from maize silage-fed (C) and hydroponic forage-fed, with 50% (LH) and 100% (HH) silage replacement.*

A)	**from/to**	**P**	**S**		**Total**	**Correct response (%)**
	P	37	8		45	82.2%
	S	3	42		45	93.3%
	Total	40	50		90	88%
B)	**from/to**	**C**	**LH**	**HH**	**Total**	**Correct response (%)**
	C	33	0	3	36	91.7%
	LH	0	36	0	36	100%
	HH	0	0	36	36	100%
	Total	33	36	39	108	97%

For the buffalo milk samples, only three misclassifications occurred for C samples, which were recognised as HH samples, leading to a 97% total correct response. Milk from buffaloes fed with hydroponic barley forage (LH and HH) was 100% correctly classified. The results show that a pasture-fed diet, compared to an indoor diet, leads to a lower % of correct classification. The more variable responses obtained for P milk could be linked to VOCs, the presence of which varies both qualitatively and quantitatively according to the fed period and, hence, the botanical composition of the pasture (Altomonte et al., 2019).

3.2 Analysis of Volatile Compounds

Compared to S milk, P milk had a higher overall quantity of VOCs in the headspace. Terpene compounds, such as α-pinene, β-pinene, β-caryophyllene and limonene, qualitatively characterised the P milk samples, while S milk had a higher quantity of hydrocarbons and ketones. Similar findings on milk VOCs from pasture-fed goats have been reported by Sant'Ana et al. (2019). Milk from buffaloes fed maize silage had a higher quantity of acids (e.g. butanoic and hexanoic) and ketones, such as diacetyl, while milk from buffaloes fed hydroponic forage had a higher amount of dimethyl sulphone, as well as 1-octen-3-ol, which can result from the metabolism of linoleic and linolenic fatty acids (Curioni and Bosset, 2002).
In conclusion, E-nose resulted a useful device for the rapid control of the genuineness of raw milk obtained from ruminants fed with the inclusion of fresh forage in the diet. It could be used to protect producers and consumers of dairy products from potential fraud.

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Use of deep learning in combination with FT-NIR spectroscopy for the analysis of extra virgin olive oil

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The first two years of the PhD project were dedicated to two main objectives i) acquiring chemical and spectral data from various categories of olive oils to develop predictive models for the chemical profile; and ii) developing NIR-based models to quantify adulterations of EVOO with seed oils. Data acquisition is currently ongoing, and preliminary results have shown the following: i) a well-distributed range of chemical profile features, which is essential for further model development, and ii) the successful calibration of Partial Least Squares (PLS) predictive models for adulteration, showing excellent performance metrics (R^2 = 0.99; near-zero BIAS; and RMSE ranging from 1.7 to 3.2).

Fattibilità di impiego dell'apprendimento profondo in combinazione alla spettroscopia FT-NIR per l'analisi dell'olio extra vergine di oliva

Nei primi due anni di dottorato sono stati i) acquisiti dati spettrali e chimici da diverse categorie di olio di oliva per lo sviluppo di modelli predittivi del profilo chimico, e ii) sviluppati modelli basati sulla spettroscopia NIR per la quantificazione di adulteranti in EVOO. L'acquisizione dati è in corso. Dai risultati preliminari si evince i) una buona distribuzione dei parametri nel profilo chimico (necessaria per lo sviluppo di un buon modello) e ii) modelli predittivi basati su PLS con metriche prestazionali eccellenti (R^2 > 0.99; BIAS tendente a 0; and RMSE tra 1.7 e 3.2).

Keywords: Olive oil, quality, adulteration, shelf-life, FT-NIR spectroscopy, chemometrics, deep learning

1. Introduction

In accordance with the PhD thesis project (Bandiera A., 2022), the present manuscript reports its main results.

A1. **Development of the research strategy:** the study includes the state-of-the-art analysis for A1.1, which involves the use of UV-Vis and NIR spectroscopy for the quali-quantitative analysis of EVOOs. It also includes A1.2, the selection of classical analytical methods for the analysis of EVOOs, and A1.3, the selection and testing of DNN algorithms for the modelling of EVOOs' spectral data.

A2. **Chemical-profile and shelf-life prediction models:** this activity involves the development of DNN-based models to assess the chemical profile and shelf-life of EVOOs. It includes A2.1, shelf-life tests; A2.2, UV-Vis and FT-NIR spectral scans, and chemical analysis of samples; A2.3, calibration of models using both classical and deep chemometrics; and A2.4, validation of models using external datasets.

A3. **Adulteration identification models:** this activity focuses on the identification and quantification of adulteration in EVOOs using DNN models. It includes A3.1, samples preparation and UV-Vis and FT-NIR scans; A3.2, calibration of predictive models; and A3.3, validation of predictive models.

2. Materials and Methods

2.1 Sample preparation

For activity A2, monthly olive oil samples from Umbria Olii International Spa, an olive oil refinery factory (Perugia, Italy) included extra virgin, virgin, lampante, pomace and refined. Chemical analytical reports were provided for each sample. Samples were stored in dark at 25 °C for 24 hours at 25 °C before spectral analysis. Samples for the activity A3 were prepared (Vanstone et al., 2018) by blending EVOO from the "Oleificio Sociale di Canino" (Viterbo, Italy) with 4 seed oils (i.e., peanut, sunflower, maize, soy) purchased from a local market. The blend concentrations for each adulterant were as follows: 0, 0.5, 1.5, 3, 5, 10, 15, 20, 60 and 100 % (w/w). Four biological replicates were prepared.

2.2 Spectra acquisition

Spectra were acquired using UV-Vis and FT-NIR spectrophotometers, mod. Lambda 850+ (PerkinElmer, USA) and mod. Antaris II (ThermoFisher, USA), respectively. The former operated within the spectral range of 380-900 nm with a resolution of 1 nm, while the latter covered the range of 1000-2500 nm with a resolution of 1.93 nm. Before scanning, the samples were filtered using fast paper qualitative filters. Spectral acquisition took place at a controlled temperature of approx. 32 °C using optical-glass cells with path lengths of 1 and 6 mm. Three technical replicates were carried out. Spectral acquisitions are still going, and currently, around 300 and 400 spectra were acquired for A2 and A3, respectively.

2.3 Descriptive statistics

The chemical-profile data from the analytical reports produced by Umbria Olii International (activity A2) were subjected to statistical analysis to verify deviations from the normal distribution for each predictand (i.e., analyte).

2.4 Calibration and cross-validation of predictive models

Partial Least Squares (PLS) models, developed using Matlab R2017b software (Mathworks, USA) and PLS toolbox v8.6.2 (Eigenvector research Inc., USA), were used for chemometrics modeling (Nallan Chakravartula S.S. et al. 2022). Deep chemometrics (DNN) models were developed using Python3 with Keras and PyTorch/TensorFlow packages. Spectral pre-treatments, including Standard Normal Variate (SNV); Multiplicative Scatter Correction (MSC); Extended MSC (EMSC); and Savitzky-Golay 1st and 2nd derivatives with a 2nd or 3rd order polynomial fitted over a window of 25-45 features, were tested alone or in combination. These pre-treatments were always followed by mean-centering (MC) or autoscaling (AS). The optimal number of latent variables (LVs) was selected using the "Venetian blind" cross-validation method. Performances were evaluated in terms of Root Mean Squared Error (RMSE), systematic error (BIAS), and R^2.

3. Results and Discussion

Results of the analysis conducted for activities A2 and A3 are presented. It is important to note that the ongoing scans of UV-Vis and FT-NIR data limits the presentation to the statistical analysis of A2 data and the calibration of A3 models.

3.1 Chemical-profile data overview

Table 1 shows the descriptive statistics of a set of analytical data acquired by the refinery factory. Additional features (data not shown) include triolein, ester fraction (cholesterol, brassicasterol, campesterol, stigmasterol, delta7-stigmasterol, delta7-avenasterol), fatty acids (lauric, myristic, palmitic, palmitoleic, stearic, arachidic, eicosenoic, behenic, erucic and lignoceric acids), and trans-fatty acids (oleic, linoleic and linolenic acids). Data had a good range for each feature, but not all of them passed the normality tests, posing a potential risk to model performances. However, sampling is ongoing, and a more representative sample size should improve this aspect. In this context, data transformation will be also tested.

Table 1 *Descriptive statistics of some analytes used in the development of PLS and DNN chemical-profile prediction models.*

Stats	Acidity (% w/w)	Peroxides (meq/kg)	Ethyl esters (mg/kg)	Waxes (mg/kg)	Total sterols (g/kg)	Oleic ac. (%)	Linoleic ac. (%)	Linolenic ac. (%)
Min	0.05	0.10	4.25	47.40	0.14	18.05	0.01	0.01
Max	15.20	12.30	27.94	542.40	11.20	76.89	0.50	0.10
Mean	3.17	7.98	12.95	238.20	1.68	68.62	0.06	0.04
St. Dev.	3.98	3.00	4.79	120.14	1.88	11.72	0.10	0.03

3.2 Adulteration prediction models

Table 2 shows the best predictive model for each adulterant selected among 140 spectral pre-treatments.

Table 2 *Summary of best PLS predictive models for adulterant quantification in EVOO.*

Adulterant	Pre-treatment				LVs[e]	RMSE (%)		BIAS (%)		R^2	
	SG[a]	Deriv.[b]	SC[c]	Norm.[d]		C[f]	CV[g]	C	CV	C	CV
peanut	25	1st	EMSC	MC	5	2.796	3.245	-1.641E-12	0.108	0.989	0.985
sunflower	29	none	EMSC	MC	2	1.735	1.833	0E+00	-0.039	0.996	0.995
maize	25	1st	EMSC	AS	2	1.910	2.010	-7.105E-15	-0.014	0.995	0.994
soy	29	none	EMSC	MC	2	1.775	1.848	5.329E-14	-0.004	0.996	0.995

a, Savitzky-Golay filter; b, derivative; c, scatter correction; d, normalization; e, latent variables; f, calibration; f, cross validation.

For all four models, the use of a SG smoothing window consisting of 25-29 features improved the signal-to-noise ratio, resulting in enhanced prediction performance. The models for peanut and maize oils exhibited notable improvement when subjected to the 1st derivative transformation. Additionally, the removal of light scattering through EMSC was crucial in all tests. Spectra normalisation using the MC proved effective in most cases, except for the maize model, which showed superior performance with AS normalization. Two LVs were captured over 95 % of the variance in all models, except for peanut model, which required five LVs. Notably, the peanut model showed a consistently higher RMSE, approx. 50 % greater than the other models. This difference can be attributed to a few possible outliers in the 60 % concentration adulteration. Further tests, including outlier analysis, are required to enhance the model precision. BIAS values across all models tended toward zero, indicating excellent accuracy. Future studies will involve the development of PLS models for the chemical profile and EVOO shelf-life tests, as well as the implementation of Deep Neural Networks in all case studies.

Colour Stability Of Rosé Wines As Affected By Phenolic Composition And Tannins Addition

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Two of the activities of the PhD thesis project are described. A first subject was the study of the colour and oxidative evolution of five model rosé wines during three weeks of storage. These were made by blending different ratios of anthocyanins and tannins in a model solution. As a second subject, 8 distinct oenological tannins were tested for their ability in modulate and stabilize the colour parameters of a rosé wine made at laboratory scale. The overall research project aims to investigate the mechanisms of formation, evolution and stabilisation of colour in rosé wines as a function of different technological intervention or storage strategies.

Stabilità del colore dei vini rosati in funzione della composizione fenolica e dall'aggiunta di tannini

Sono descritte due delle attività del progetto di tesi di dottorato. Un primo argomento è stato lo studio dell'evoluzione cromatica e ossidativa di cinque vini rosati modello durante tre settimane di conservazione. Questi sono stati ottenuti miscelando diversi rapporti di antociani e tannini in una soluzione modello. In secondo luogo, sono stati testati 8 tannini enologici distinti per la loro capacità di modulare e stabilizzare i parametri del colore di un vino rosato prodotto su scala di laboratorio. Il progetto di ricerca complessivo mira a indagare i meccanismi di formazione, evoluzione e stabilizzazione del colore nei vini rosati in funzione di diversi interventi tecnologici o strategie di conservazione.

Key words: oxidation, rosé wine, anthocyanins, oenological tannins, polyphenols, shelf life .

1. Introduction

The colour of rosé wines varies widely; for example, the colour density (CD) may vary from 0.30 AU in the lightest rosés to 1.40 AU in the darkest rosés, as does the tint (H) from 1.50 to 0.90 (Leborgne et al., 2022). These variabilities depend on both the grape cultivar and the winemaking procedures which eventually affect the acceptance and visual quality of the product. However, little is known about the impact of the different phenolic/pigments ratio on the attitude of a rosé wine to maintain its overall colour and oxidative balance. Furthermore, another matter of concern is how the exogenous addition of oenological tannins may affect the rosè wine attributes as they can influence the colour, helping to achieve the desired shade and brilliance of the wine. Tannins could also contribute to the wine stability, preventing oxidation and impacting the long-term colour expression and shelf-life durability of rosé wines.

2. Materials and Methods

For the A1 purpose, five distinct rosé nuances were obtained by mixing solutions of oenocyanin and grape tannins at different concentrations in a 12% V/V hydroalcoholic model solution containing tartaric acid and Fe (II) (5 mg/L) and stored in 50mL bottles. Depending on the ratio between oenocyanin and tannins, five different model rosé wines were obtained: VD (Very Dark), D (Dark), I (Intermediate), L (Light) and VL (Very Light). Oxidation was ignited by adding 200µM of hydrogen peroxide and bottles were kept in a cool and dark place during 20 days of storage. The analyses aimed at the quantification of the main wine's parameters related to the phenolic and the colour, together with the iron speciation and the oxidative evolution over time. Regarding the A2 purpose, this research is currently taking place at the faculty of pharmacy of the University of Sevilla (Spain). Eight tannins of different origins were separately added to a rosé wine made at laboratory scale from sangiovese and merlot grapes. All the oenological tannins were previously characterized to determine their phenolic richness (Vignault et al. 2018). The OCR (Oxygen Consumption Rate) was also evaluated, according to the non-invasive luminescence technique described by Pascual et al., 2017. Transparent glass bottles (0.30 L) equipped with a patch to measure dissolved oxygen were used. The experiments were conducted in a model wine solution composed of 10% ethanol, 4 g/L tartaric acid and pH adjusted to 3.5. This was supplemented with 5 mg Fe (III)/L in the form of iron chloride hexahydrate.

3. Results and Discussion

3.1 Determination of the colour evolution over time

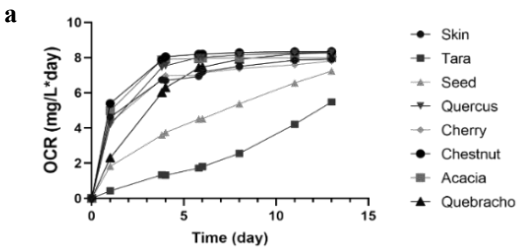
The evolution of the phenolic component and the anthocyans content was studied over time, although particular attention was paid to the oxidation evolution of the solutions, as these were subjected to the Fenton reaction speeded up by adding hydrogen peroxide. The colour of the solutions changed differently over time, depending on their initial phenolic content. The colour change was observed in the different solutions; yellower nuances increased considerably compared to T0, whereas the coordinate a* decreased over time (Table 1). The increase in the b* coordinate was one of the main causes of the decrease in a* in all our samples, meaning that the final solutions absorbed at 420nm more than the T0 solution. HPLC analyses aimed to study the individual anthocyanins evolution over time, and they yielded interesting results. For example, it has been observed that specific anthocyanins degrade much faster than others and differently from one sample to another. Delphinidin-3-glucoside degraded the most and the fastest three weeks after the first sampling, remaining at levels of less than ten per cent compared to T0. On the opposite, vitisin A demonstrated to be highly stable to oxidation, undergoing to limited diminution over time. As a result of the analysis carried out it has been shown that in most cases the presence of a higher amount of anthocyanins seems to have a positive influence on the prevention of wine oxidation. Indeed, it seems that lighter-coloured wines tend to change colour more quickly, as a signal of faster oxidation.

	VD		D		I		L		VL	
CD	22.30 ± 5.68	a	31.47 ± 8.33	a	68.96 ± 13.88	b	77.9 ± 14.43	b	121.95 ± 11.62	c
dA%	-26.53 ± 2.83	a	-50.94 ± 2.96	ab	-73.46 ± 2.89	ab	-100.00 ± 0.00	b	-100.00 ± 0.00	b
Polymers contribution%	26.43 ± 0.57	a	19.34 ± 0.47	b	16.38 ± 0.06	c	8.95 ± 0.14	d	2.61 ± 0.27	e
L*	11.59 ± 2.29	a	4.20 ± 3.12	b	0.37 ± 2.47	b	-1.29 ± 2.16	b	-2.09 ± 3.84	b
a*	-23.18 ± 1.98	a	-26.63 ± 1.42	a	-18.98 ± 2.31	ab	-20.79 ± 1.68	b	-4.38 ± 1.62	c
b*	311.96 ± 5.24	a	365.42 ± 4.81	b	250.36 ± 4.56	c	228.91 ± 4.28	cd	213.29 ± 4.12	d
C	-11.21 ± 2.03	a	8.02 ± 2.54	b	79.19 ± 2.35	c	122.62 ± 3.52	d	175.48 ± 3.27	e
H	-69.33 ± 0.81	a	-52.01 ± 0.42	b	-34.85 ± 0.76	c	-25.68 ± 0.48	d	-19.98 ± 1.02	e
TPI	-10.80 ± 3.86	a	-13.59 ± 3.46	a	-17.89 ± 0.61	b	-22.72 ± 0.73	c	-13.77 ± 1.42	a
VRI	-15.69 ± 0.96	a	-19.38 ± 1.43	a	-18.30 ± 0.67	a	-13.46 ± 1.58	a	-16.16 ± 4.59	a
Total anthocyans mg/L	-51.67 ± 2.81	a	-50.57 ± 4.12	a	-50.63 ± 5.01	a	-41.70 ± 3.05	ab	-32.46 ± 5.59	b

Table 1 *Changes in colour and phenolic parameters (as percentages) of the distinct model rosé wines at T480 compared to T0.*

3.2 Characterization of oenological tannins useful to stabilize the colour of rosé wines

The oxygen consumption kinetics of the different oenological tannins in an oxygen-saturated model wine solution was studied at 20°C (Figure 1a). The seed and tara tannins consumed oxygen slower than tannins from other sources; on the other hand, it is evident how the others followed a similar trend until they stabilised after about 8 days of storage because of the total consumption of dissolved oxygen. Tannins that consume oxygen the fastest are the most effective in terms of protecting the wine from chemical oxidation (Vignault et al., 2018). According to Pascual et al., 2017, of the various oenological tannins, ellagitannins are the fastest oxygen consumers, followed in decreasing order by quebracho tannins, skin tannins, seed tannins and finally gallotannins. Once the tannins were characterized, they were added at 200 mg/L to a rosé wine. The capacity of different tannin to chelate iron in the samples was studied. Figure 1b illustrates the varying degrees to which the different tannins chelate the iron present in the wine samples over a period of two months. Our findings indicate that chestnut tannin exhibited the highest efficiency in reducing total iron levels in the wine; it was found to consume approximately 20% of the initial iron concentration at T0. In conclusion, the first results of this study indicate some interesting properties of tannins depending on their botanical origins. Chestnut tannin, in particular, seems to have promising features in view to promote colour stability in the production process of rosé wines.



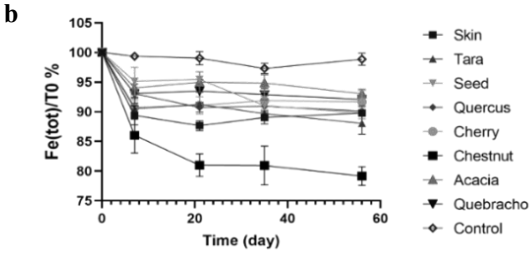


Figure 1a *Oxygen consumption kinetics of eight different oenological tannins in an oxygen-saturated model wine solution at about 20°C*

Figure 1b *Iron chelation kinetics of the eight sample wines containing the different oenological tannins and control over time, compared to T0.*

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Novel plant protein sources for the beverage sector: technological functionality, nutritional properties, sensory characteristics, and consumer acceptability

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The aim of this Ph.D. research is to identify plant proteins (PP) suitable for the beverage sector by filling the knowledge gap about their technological and sensory performance. The first 3 activities of the Ph.D. project are: (i) development of a database on PP functionalities, through literature research and benchmark analysis, (ii) selection of PP samples and assessment of their suitability as beverage ingredients, by analyzing cold and hot water solubility and sensory properties; (iii) preliminary prototyping of a coffee beverages containing selected PP samples.

Nuove fonti proteiche vegetali per il settore delle bevande: funzionalità tecnologiche, proprietà nutrizionali, caratteristiche sensoriali ed accettabilità del consumatore

L'obiettivo di questo percorso di dottorato è quello di identificare proteine vegetali (PP) impiegabili nel settore delle bevande, colmando la carenza di informazioni relative alle loro proprietà tecnologiche e sensoriali. Le prime 3 attività del progetto sono: (i) sviluppo di un database sulle funzionalità delle PP attraverso una ricerca bibliografica e di benchmark; (ii) selezione di ingredienti proteici e valutazione della loro attitudine ad essere impiegati come ingredienti di bevande, analizzandone la solubilità in acqua calda e fredda e le proprietà sensoriali; (iii) realizzazione di prototipi di bevande a base di caffè contenenti gli ingredienti proteici selezionati.

Keywords: Plant proteins, solubility, sensory analysis, beverage, coffee.

1. Introduction

In accordance with the Ph.D. thesis project previously described (Barozzi, 2022a), this poster reports the main results of the activities concerning: (A1) Development of a database relevant to plant protein (PP) functionalities; (A2) Analysis of selected PP ingredients, chosen based on A1; (A3) Preliminary prototyping of a PP coffee-based beverage using ingredients selected based on A2.

2. Materials and Methods

A1 Development of a database relevant to plant protein functionalities
A literature review was conducted on Web of Science, Scopus, and Google Scholar to collect information on PP food-related properties. Information retrieved from more than 200 research papers was comprehensively organized in a database that collected data relevant to PP sources, extraction method, extraction yield, extract protein content, technological and sensory properties, nutritional characteristics, allergenicity, consumer acceptability, and regulatory issues. Further information about market availability and current application in beverages was obtained thanks to a benchmark analysis performed in collaboration with Lavazza using the Mintel database.
A2 Analysis of plant protein ingredients selected based on A1
PP samples, selected based on the database developed in A1, were kindly provided by Lavazza. The total protein content of these samples was evaluated through bicinchoninic acid assay (BCA). Samples were also assessed for solubility in cold water. To this aim, PP samples (0.2 g, W) were stirred for 6 h in 20 mL water at 20 ± 1 °C. The dispersions were centrifuged, and the sediment was dried and weighed. The protein content of the supernatant was analyzed through BCA (P) and used to estimate protein solubility (S_p), which was expressed as:

$$S_p = P/W \cdot 100 \quad (1)$$

The flavour acceptability of the PP samples was assessed by 12 judges with previous experience in sensory analysis. Cold water solubilized samples (30 mL, 1% w/w) were provided to the judges, who were asked to drink the samples and evaluate flavor acceptability on a 7-point scale anchored at 1 (unacceptable), 4 (neutral), and 7 (desirable). Samples selected based on cold-water solubility (S_p) and sensory analysis were analyzed for solubility in hot water. To this aim, 0.5 g of protein sample were inserted in a coffee filter (Melitta, Minden, Germany) and washed with 8.3 mL hot water (96 ± 1 °C). The percolated aqueous solution was analysed for total protein and protein solubility.
A3 Preliminary prototyping of a PP coffee-based beverage using protein ingredients selected based on A2
PP selected based on A2 results, were assessed for their suitability as ingredients in a simplified prototype

represented by a ground coffee-based powder intended for the preparation of a beverage by hot water percolation. To this aim, PP samples were mixed at increasing concentration (0, 2, 5, 10, and 30% w/w) with 3 coffee blends having different roasting degrees. The PP-coffee mixtures (15 g) were inserted in coffee filters and washed with 240 mL hot water (96 ± 1 °C). The aqueous extracts were characterized in terms of dry matter, protein content, and protein recovery (percentage of proteins in the extract as compared to the initial protein content in the PP-coffee mixture).

3. Results and Discussion

A1 Development of a database relevant to plant protein functionalities

Fig.1 schematically represents the rationale of the experimental plan applied to select PP samples suitable for beverage production. Among the 84 PP sources initially identified by the literature review, 30 were selected based on their high solubility, no allergenicity, and availability on the market. These PP samples included protein concentrates, isolates, hydrolysates as well as protein samples prepared from pea residues (Barozzi et al., 2022b).

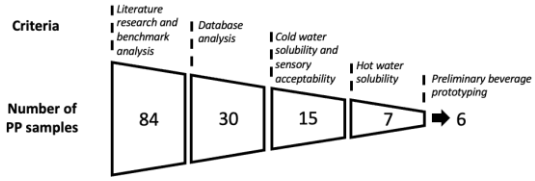

Figure 1. Schematic representation of the criteria adopted for the selection of plant protein (PP) samples suitable for beverage production.

A2 Analysis of plant protein ingredients selected based on A1

The total protein content of the selected PP, ranged from 12 to 83% w/w. Protein solubility **(S_p)** in cold water ranged from 5 to 52% w/w. Among all samples, protein hydrolysates having the lower molecular weight accounted for the highest protein solubility (Beaubier et., 2021). The peculiar vegetable notes of PP might hinder their use as food ingredients (Kumar et al., 2022). In fact, sensory analysis evidenced only 15 samples out of the original 30 to present an acceptable flavor. These selected samples were subjected to hot water extraction to have a first insight into their suitability as beverage ingredients. Filter clogging was observed in 8 samples, probably due to protein coagulation under the applied high-temperature conditions (Ge et al., 2021). In the remaining 7 PP samples, no clogging was observed, associated with a relatively high protein solubility. These samples were thus selected to perform preliminary beverage prototyping activities (A3).

A.3 Preliminary prototyping of a PP coffee-based beverage using protein ingredients selected based on A2

The feasibility of the 7 samples selected during A2, as possible ingredients of a PP-enriched coffee powder intended for hot beverage preparation was assessed. The roasting degree of the coffee blend had no effect on protein extraction. As expected, the higher the PP:coffee ratio in the powder mixture, the higher the protein content in the final beverage. Although no filter clogging was registered in any sample, one of them was not considered for further analysis, due to its lower solubility in the considered prototyping conditions.

4. Conclusion and future activities

A1. The developed database, accounting for a full range of functionalities and market factors, is a powerful tool for the identification of protein ingredients with the desired characteristics. **A2.** PP ingredients suitable for beverage production were identified based on hot water protein solubility and sensory acceptability. **A3.** No effect of the coffee blend was observed on the solubility of the PP ingredients, suggesting the technical feasibility of coffee-based hot beverages enriched with PP.

The next activities will be focused on the optimization of PP-coffee powder mixtures, with the aim of identifying sensory pleasant formulations feasible for the preparation of beverages *via* hot water percolation. This activity is being performed during the 6 months secondment at Lavazza Innovation Center.

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ECO-sustainable packaging materials for the food industry (ECOPACKMAT)

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The activities described so far in the doctoral program have been completed. Specifically, I worked on the optimization of a multi-step cellulose extraction method by reducing its environmental impact, as well as on its standardization for different tested lignocellulosic biomasses. The cellulosic residue collected was systematically characterized via nuclear magnetic resonance (NMR) and with different magnification at the optical microscope. The production of nanostructure including cellulose nanocrystals (CNCs) was also successful, and the dimensional aspects thereof analyzed with dynamic light scattering technique (Particle Size Distribution), and atomic force microscopy (AFM).

Miglioramento del metodo di estrazione e caratterizzazione della cellulosa e dei nanocristalli

Le attività descritte fino ad ora nel programma di dottorato sono state concluse. Nello specifico, ho lavorato sull'ottimizzazione di un metodo multi-step per l'estrazione di cellulosa attraverso una riduzione dell'impatto ambientale associato, cercando altresì di standardizzarne le varie fasi per le diverse biomasse lignocellulosiche testate. Il residuo cellulosico raccolto post-estrazione è stato sistematicamente caratterizzato per quanto concerne composizione (mediante risonanza magnetica nucleare – NMR) e morfologia (mediante microscopia ottica). E' stata inoltre effettuata con successo l'estrazione di nanocristalli di cellulosa (CNCs), caratterizzati per quanto concerne morfologie e dimensione mediante microscopia a forza atomica (AFM) e dynamic light scattering (DLS).

Keywords: Cellulose, cellulose nanocrystals (CNCs), food packaging, nuclear magnetic resonance (NMR), circular economy.

1. Introduction

In accordance with the research plan of the PhD work, here are presented the outcomes of the initial two work packages (WP) and related tasks regarding:

(WP1) Cellulose extraction from different lignocellulosic biomasses and NMR characterization, production of cellulose nanocrystals from cellulosic residue and characterization thereof through DLS and AFM techniques.

(WP2) Bulk addition of CNCs into paper-based materials.

2. Materials and Methods

This work starts with the extraction of cellulose from several lignocellulosic biomasses (e.g., giant-cane cut up, *Posidonia oceanica*, and coffee silverskin) and finishes with the production of CNCs. Cellulose recovery was performed via a multi-step procedure previously described in our recent work. Briefly, the partial removal of hemicellulose and lignin was obtained using a sodium hydroxide solution upon stirring at room temperature. Secondly, xylene was applied to remove small MW organic compounds. Finally, a bleaching treatment with sodium chlorite washed out both pigments and lignin residues. Each extraction step was followed by washing with water, ethanol or acetic acid, filtration, and further drying in an oven at 105°C. The so-obtained cellulosic residue represented the input for the CNCs production. The latter involved the treatment of the solid with a strong sulfuric acid solution, anticipated by a homogenization step needed to boost the acid penetration inside the cellulosic core. Afterward, the solid-liquid separation was achieved by centrifugation, whereas the pH of the obtained CNCs was raised up to 4.5 through several washing steps. At last, pH was adjusted to neutrality using a dialysis tube against deionized water.

3. Results and Discussion

3.1 Determination of the yield and characterization of cellulose

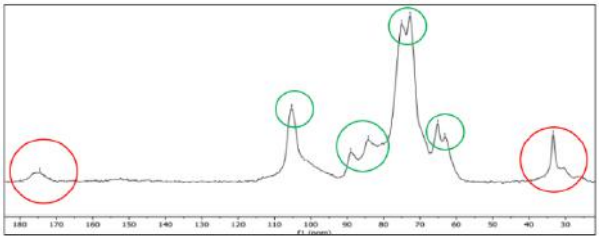
Table 1 displays the results of cellulose extraction yield obtained in this work, together with those already observed by other authors in the literature when testing the same biomasses.

SUBSTRATE	Experimental cellulose yield (% dry basis)	Cellulose yield from literature (% on dry basis)
Giant-cane cut up	~38.60	~38.20
Posidonia Oceanica	~43.10	~40.20
Coffee silverskin	~23.40	~23.80

***Table 1**: Comparison between yields of cellulose extraction found in this work and those retrieved from literature.*

The slight difference detected between the current results and those from the literature (Table 1) can be ascribed to the presence of some impurities (e.g., lignin and hemicellulose residues), as clearly visible from the NMR spectrum pertaining to the cellulose recovered from Posidonia Oceanica (Figure 1). Therefore, further efforts must be made in the nearby future to eventually increase the cellulose purity inside the final residue.

***Figure 1**: NMR spectrum of Posidonia Oceanica (the red circles represent the impurities, lignin and hemicellulose, while the green circles belong to the cellulose carbon atoms).*



3.2 Percentage of CNCs and characterization

According to the morphological study, the final particles obtained after acid hydrolysis cannot properly be defined as CNCs because of a size slightly bigger than the 'nano' scale as supported by the acquisition of the AFM images (Figure 2) and consistent with the data from the particle analyzer (Figure 3).

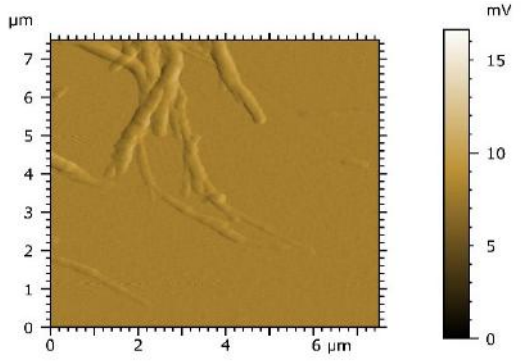


***Figure 2**: AFM image of nanostructure*

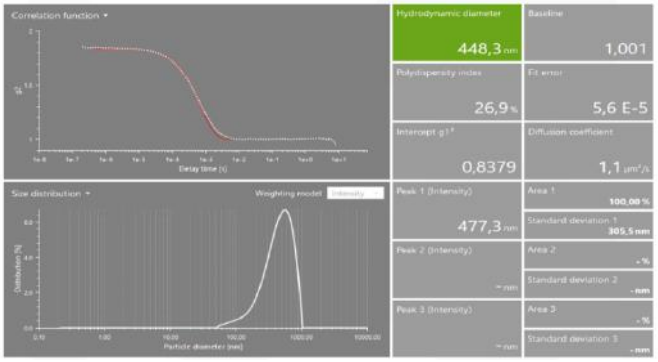


***Figure 3**: software image of particle analyzer after analysis of nano structure solution*

3.2 Preparation of cellulose sheet and bulk insertion of CNCs

In this frame, I would like to impregnate the cellulose sheet with some water-proof compounds (e.g., natural waxes), as well as to improve the oxygen barrier properties thereof via addition of CNCs. However, this task is still at the early developing stages, despite I found interesting research articles that can be helpful to reach the goal within the next year.

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Cow's Milk and Plant-based Beverages: a comparison of their effect on markers of human health and on nutrient intake

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The first two activities of the PhD thesis project are described. Firstly, a systematic review of the impact of substituting cow's milk (CM) with plant-based drinks (PBD) on markers of human health was performed. Secondly, the impact on nutrient intake following the substitution of CM with PBD in two Mediterranean dietary patterns, one based on the Italian Dietary Guidelines (IDG) and the other in line with the "Planetary Diet" and adapted to the Italian food habits (EAT-IT), was assessed.

Il latte e le bevande vegetali: comparazione dei loro effetti su marker della salute e sull'assunzione di nutrienti

Le prime 2 attività del progetto di tesi di dottorato sono descritte. È stata svolta una revisione sistematica riguardante l'impatto della sostituzione del latte con le bevande vegetali sui marker della salute. Successivamente, è stato valutato l'impatto sull'intake di nutrienti a seguito della sostituzione del latte con le bevande vegetali in due piani alimentari mediterranei, uno basato sulle linee guida italiane e l'altro in linea con la "Dieta Planetaria" adattata alle abitudini alimentari italiane.

Key words: Cow's milk, plant-based alternatives, dietary patterns, and health, nutrition, sustainability.

1. Introduction

In accordance with the PhD thesis project previously described (Biscotti, 2021), this poster reports the main results of the first two activities concerning:

(A1) the analysis of data in literature regarding the effects of the CM substitution with PBD on markers of human health through a systematic review;

(A2) the assessment of the impact on nutrient intake due to the substitution of CM with PBD both within a dietary pattern based on the Italian Dietary Guidelines (IDG) (CREA, 2018) and an Italian-Mediterranean dietary pattern based on the EAT-Lancet Reference Diet (EAT-IT) (Tucci *et al.,* 2021).

2. Materials and Methods

(A1) A systematic literature search was conducted using two digital databases PubMed® and Scopus. The search was performed in May 2022 and updated in July 2022. Studies were included if they were clinical trial tested the substitution of CM with PBD on markers of human health. There were restrictions pertaining to age (<18 years within the target population), but not to other characteristics of study participants (e.g., BMI and health condition).
(A2) The list of ingredients and the nutrition declaration of PBD were collected from home-shopping website of the retailers present on the Italian market. Selected PBD were grouped into six categories: almond drinks; blends (≥2 plant-based ingredients); oat drinks; rice drinks; soy drinks; other single ingredient (e.g., coconut drinks). Then, the PBD categories were divided in calcium-fortified (Ca) and not-calcium fortified (nCa) products. The substitution of CM within IDG and EAT-IT dietary patterns was made with the average nutritional value declared on the food labels of 309 retrieved PBD. The elaboration of dietary patterns and the estimation of the nutritional composition of the different dietary plans were made by MetaDieta Software.

3. Results and Discussion

3.1 The effect of cow's milk substitution with PBD on markers of human health

A total of 29 papers were collected; 27 studies focused on soy drink (SD) (one of which included two trials and one of which also evaluated the effects of almond drink) while 2 studies focused on rice drink. However, it's important to note that, on the market, there are at least 20 different PBD derived from cereals, legumes, nuts, pseudo-cereals, and seeds.
The most investigated parameters following the CM substitution with SD were anthropometric measurements (n = 13), lipid profile (n = 8), markers of inflammation and/or oxidative stress (n = 7), glucose and insulin response (n = 6) and blood pressure (n = 4). A comparison of the findings of the studies included was difficult due to variability in terms of doses, nutritional composition of PBD and CM, study design, characteristics of the recruited subjects, duration, and markers. However, the results of this systematic review seem to suggest a potential protective role of SD on lipid profile since five of the eight studies included showed that consumption of SD compared to CM resulted in LDL-C lowering effect. Regarding the other health-related outcomes, no differences

of interest between the consumption of SD and CM were found.
Since only one study focused on bone health and also because the main nutritional differences between CM and PBD are related to Ca and Vit. D, which have a wide impact on bone health, future studies should focus also on their impact on markers of bone health (e.g., bone mineral density). In conclusion, further studies are needed to better elucidate the effect of substituting CM with PBD in different target of groups of the population, especially in the long term.

3.2 The effects of cow's milk substitution with PBD on nutrient intake

The analysis of nutrient intake showed that substitution of CM with all PBD had the same effects in both IDG and EAT-IT dietary patterns. In terms of macronutrients, the substitution of CM with all-PBD resulted in a reduced intake of protein, saturated fat (SFA), cholesterol, and increased intake of fiber. The amount of total fat, carbohydrates and sugars was dependent on the type of PBD; the detailed differences are shown in Fig. 2.

			Total fat	Total carbohydrates	Sugars
Ca-FORTIFIED	EAT-IT	Ca-almond	-	-	-
		Ca-blends	-	+	-
		Ca-oat	+	+	-
		Ca-rice	-	+	+
		Ca-single	+	-	-
		Ca-soy	+	-	-
	IDG	Ca-almond	-	-	-
		Ca-blends	-	+	-
		Ca-oat	+	+	-
		Ca-rice	-	+	+
		Ca-single	+	-	-
		Ca-soy	+	-	-

			Total fat	Total carbohydrates	Sugars
nCa-FORTIFIED	EAT-IT	nCa-almond	+	-	-
		nCa-blends	+	+	=
		nCa-oat	-	+	-
		nCa-rice	-	+	+
		nCa-single	+	+	-
		nCa-soy	+	-	-
	IDG	nCa-almond	+	-	-
		nCa-blends	+	+	=
		nCa-oat	-	+	-
		nCa-rice	-	+	+
		nCa-single	+	+	-
		nCa-soy	+	-	-

Figure 2 *Specific differences on macronutrients between EAT-IT and IDG with Ca-PBD and nCa-PBD compared to CM-EAT-IT and CM-IDG.*

Regarding micronutrients, all PBD-dietary patterns provided a lower amount of Vit. B_1, Vit. B_2 and Vit. B_{12} compared to both CM-dietary patterns. The intake of other micronutrients was different based on the type of considered PBD; these differences are shown in Fig. 3. In detail, the replacement of CM with all nCa-PBD resulted in a reduced intake of Vit. D and Ca. Since fortification with Ca is often combined with Vit. D, after replacement with Ca-PBD the amount of Vit. D was higher in comparison to both CM-dietary patterns. In fact, the fortification used in PBD are up to 1,5 µg/100 mL of Vit. D; however, it's important to highlight that the CM used within both dietary patterns was not fortified with Vit. D even if its fortification is increasingly present in CM on the market.
Following the replacement with Ca-PBD, the amount of Ca was dependent on the type of considered Ca-PBD. These differences are due to a variable fortification of PBD.

			Vit. D (cholecalciferol, ergocalciferol)	Calcium	Sodium
Ca-FORTIFIED	EAT-IT	Ca-almond	+	=	+
		Ca-blends	+	-	+
		Ca-oat	+	=	-
		Ca-rice	+	+	-
		Ca-single	+	=	+
		Ca-soy	+	+	-
	IDG	Ca-almond	+	=	+
		Ca-blends	+	-	+
		Ca-oat	+	=	-
		Ca-rice	+	+	-
		Ca-single	+	=	+
		Ca-soy	+	+	-

			Vit. D (cholecalciferol, ergocalciferol)	Calcium	Sodium
nCa-FORTIFIED	EAT-IT	nCa-almond	=	-	-
		nCa-blends	=	-	-
		nCa-oat	-	-	-
		nCa-rice	=	-	-
		nCa-single	=	-	-
		nCa-soy	-	-	-
	IDG	nCa-almond	=	-	-
		nCa-blends	=	-	-
		nCa-oat	+	-	-
		nCa-rice	=	-	-
		nCa-single	=	-	-
		nCa-soy	+	-	-

Figure 3 *Differences on micronutrients between EAT-IT and IDG with Ca-PBD and nCa-PBD compared to CM-EAT-IT and CM-IDG.*

It is important to consider that, despite fortified PBD contain considerable amount of micronutrients (e.g, Ca and Vit. D), their availability could still be poor (Aydar *et al.,* 2020). Therefore, unaware substitution could lead to unintended nutritional consequences due to a reduced intake of micronutrients among, for example, individuals for whom the consumption of milk may be decisive to reach the peak bone mass. However, the role of PBD should be deepened to promote a reduced intake of SFA and cholesterol in specific target of population and to reduce the environmental impact of diet. Therefore, future studies aimed to optimize dietary plans including CM and/or PBD in order to maximize diet quality minimizing their environmental impact are necessary.

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Antioxidant compounds formed in Maillard reaction of glucose and glycine

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The identification of some antioxidant Maillard reaction products has been carried out in the first part of the PhD. Some MRPs have been reported as antioxidants, although their identification remains still unclear. Therefore, the study aimed to monitor their evolution during heating a model solution of glucose and glycine. NMR was used to monitor the transient changes of MR reactants and products during time. The antioxidants were studied using HPLC coupled with a triple detector. Three antioxidant MRPs have been identified: 2-acetylpyrrole and dehydrate 1- and 3-deoxyglucosones. The antioxidants have been included in the updated kinetic model developed in literature.

Molecole antiossidanti prodotte dalla reazione di Maillard su una soluzione di glucosio e glicina

È stata effettuata l'identificazione di alcuni prodotti antiossidanti della reazione di Maillard. La loro attività antiossidante è nota, ma l'identificazione rimane incompleta. Pertanto, questa prima parte del dottorato si proponeva di studiare l'evoluzione dei MRP antiossidanti durante il riscaldamento di una soluzione modello di glucosio-glicina. L'NMR è stato utilizzato per monitorare i cambiamenti di reagenti e prodotti della MR nel tempo. Gli antiossidanti prodotti sono stati studiati mediante HPLC accoppiato a triplo detector. Sono stati identificati tre principali MRP antiossidanti: il 2-acetilpirrolo e 1- e 3-deossiglucosone deidratati. L'evoluzione di questi è stata inclusa nel modello cinetico aggiornato sviluppato in letteratura.

Key words: Antioxidants, Maillard reaction, high resolution mass spectrometry, coulometric detector, nuclear magnetic resonance, kinetic modelling

1. Introduction

In accordance with the PhD thesis project presented, this poster reports the main results of the first activities concerning:

(A1) the identification and quantification of antioxidant MRPs produced in a solution of glucose and glycine heated at 90°C for 4 hours;

(A2) the update of a kinetic model of the reaction developed in literature (Martins and Van Boekel, 2005) with the production of antioxidants.

2. Materials and Methods

The study has been developed starting from the model of the reaction proposed by van Boekel (Martins and Van Boekel, 2005), which species (glucose, glycine, acetic acid, formic acid and melanoidins) and their transient changes were monitored (NMR) and spectrophotometric assays. Then, using a novel approach based on (Ding *et al.*, 2022), which consisted in HPLC coupled with three different detectors, antioxidant MRPs were studied. The detectors used are: a diode array (DAD), a coulometric array (CoulArray™: CAD), which is selective towards antioxidants, and high resolution mass spectrometer (HRMS). DAD allowed the detection of the molecules present in our samples and the optimization of their separation through the chromatographic column. CAD, which is a multi-channel electrochemical detector, measures the current signals generated from antioxidant analytes that enter the detector. Since coulometric analyses are principally governed by Faraday's law, it allowed the quantification of the analytes, more as well as their detection. This consequentially allowed the update of the kinetic model of the reaction. Using HRMS, then, it was possible to identify the antioxidants previously detected.

3. Results and Discussion

3.1 Antioxidant activity of MRPs and their identification

Antioxidant MRPs have been detected, identified and quantified using Coularray detector and HRMS. In Figure 1(a) three main peaks have been detected and they corresponded to 2-acetylpyrrole and deydrathed version of 1- and 3- deoxyglucosones, as shown in Figure 1(b).

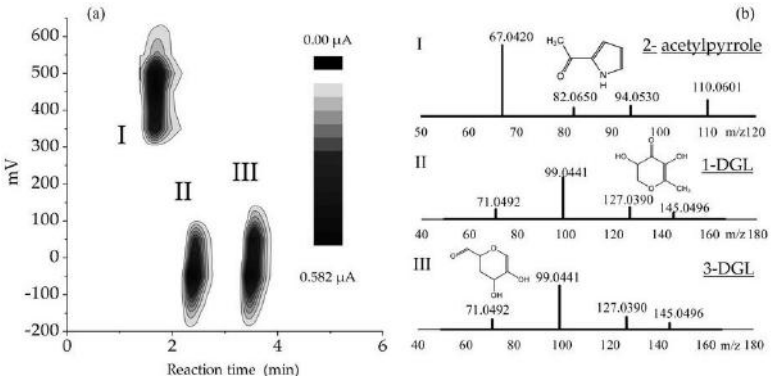

Figure 1 *:(a) Contour plot of CAD signal (sum of the signals obtain from the 16 channels) of glucose-glycine MR sample after 150min at 90°C.*

3.2 Kinetic model update

NMR spectroscopy has been used to quantify reactants (glucose and glycine) and some products (acetic acid and formic acid) along the reaction, to check if their kinetics are the same as the ones obtained by Van Boekel. In Figure 2 and Figure 2 are shown the ^{1}H-NMR spectra of acetic acid and formic acid. In details: both the signals are increasing along the MR, which let us conclude that both of them are produced during the MR, confirming the literature (Martins and Van Boekel, 2005).

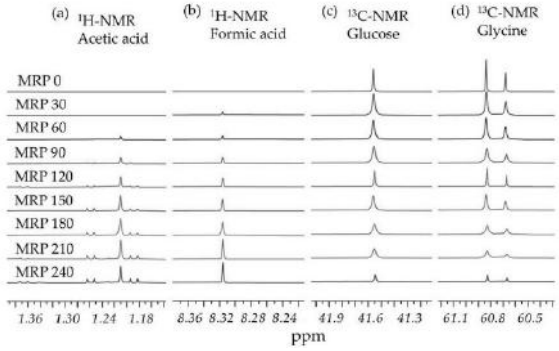

Figure 2 *:(a) and (b) Stacked ^{1}H-NMR peak corresponding to acetic acid and formic acid obtained analyzing MR samples incubated for 0, 30, 60 … 240 minutes at 90°C; (c) and (d) Stacked ^{13}C-NMR peak corresponding to glucose and glycine obtained analyzing MR samples incubated 0, 30, 60 … 240 minutes at 90°C.*

Starting from this model and including the results obtained regarding the antioxidants developed during the reaction and their mechanism of production confirmed with the literature (George and Milton, 1983)(Hayas, Bong Kim and Kato, 1985), it was possible to update the kinetic model of the reaction as reported in Figure 3.

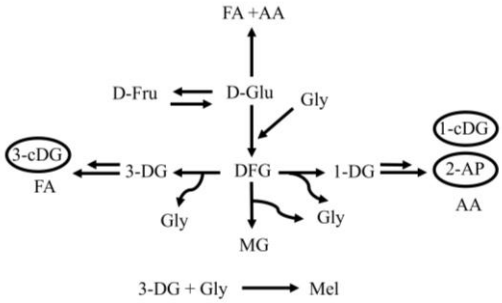

Figure 3*: Updated model of MR based on Van Boekel's one. Circled in blue, the species added.*

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By-products from agri-food industrial sector: resource or waste? An eco-friendly utilization to preserve the food's quality

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Coffee silverskin, coffee roasting by-product, was characterised for its antioxidant properties. Different extraction parameters were tested to recover bioactive compounds. The best selected extract was used in the formulation of gummy candies at different concentrations and qualitative analysis were carried out up to 120 days of storage at 25°C. Higher quality, in terms of antioxidant activity, sensory and textural characteristics, was observed in candies formulated with 1% of coffee silverskin extract. This valorisation of coffee by-product could satisfy consumer's demand for the use of eco-friendly resource to produce foods and to preserve their quality.

Sottoprodotti del settore agroalimentare: rifiuti o risorse? Un'utilizzazione eco-sostenibile per preservare la qualità degli alimenti

Coffee silverskin, sottoprodotto della torrefazione del caffè, è stato caratterizzato per le sue proprietà antiossidanti. Sono stati testati differenti parametri di estrazione al fine di recuperare composti bioattivi. Il miglior estratto è stato utilizzato, a differenti concentrazioni, per la formulazione di caramelle gommose sulle quali sono state condotte analisi qualitative per 120 giorni di conservazione a 25 °C. Le caramelle con 1% di estratto hanno presentato una spiccata qualità per attività antiossidante, caratteristiche sensoriali e strutturali. Questa valorizzazione del sottoprodotto del caffè potrebbe soddisfare la domanda dei consumatori riguardo l'utilizzazione di risorse eco-sostenibili per produrre alimenti e preservarne la qualità.

Key words: Agri-food waste; coffee by-products; bioactive compounds, fortified food, gummy candies.

1. Introduction

In accordance with the PhD thesis project, this poster reports the main results of the following activities:
(A1) Extraction of bioactive compounds from coffee silverskin and characterization of obtained extracts;
(A2) Formulation of gummy candies with selected coffee silverskin extract;
(A3) Determination of physical, chemical, sensory and microbiological characteristics of gummy candies over time.

2. Materials and Methods

Conventional (maceration, ME) and innovative (ultrasound assisted, UAE) techniques as well as variables that affect the extraction process (method, time, temperature and nature of the solvent) were tested in order to identify the best conditions to recover bioactive compounds from coffee silverskin (CS). All the extractions were tested by mixing 2 g of CS powder (moisture < 10%) with 20 mL of food grade solvents (H_2O and H_2O/ EtOH 90/10, 80/20, 70/30, 50/50 and 20/80) at different temperatures (40 °C, 50 °C, 60 °C and 70 °C) and times (30, 60, 90 and 120 minutes). Frequency of 59 kHz was used for UAE. All obtained extracts were evaluated for total phenolics content (TPC), antioxidant activity (DPPH and ABTS assays) (Costa *et al.*, 2014) and microbial count. Subsequently, the best extract (CSE) was selected for bioactive content and tested on chemical, physical (Cedeño-Pinos *et al*., 2020), microbiological (Teixeira-Lemos *et al*., 2021) and sensory characteristics of the fruit gummy candies. Control Candies (CTR) were formulated with 31% of sucrose, 28% of glucose syrup, 22% of apricot juice, 8% of pork gelatine, 1% of citric acid and 10% of water. The fortified gummy candies were formulated partially substituting water with 1% (CS1), 2% (CS2) and 4% (CS3) of CS extract. The candies were stored for 120 days at 25 °C. Statistical analysis was performed by one-way analysis of variance and Tukey's comparison test.

3. Results and Discussion

3.1 Characterization coffee silverskin extracts (CSE)

The best extract was obtained by ME at 60 ºC for 60 minutes using the hydroalcoholic mixture EtOH 30%, which showed a TPC of 1955 µg GAE mL^{-1}, 10.74 and 3.67 µmol Trolox mL^{-1} respectively for ABTS and DPPH assay. Temperatures higher or lower 60 ºC, solvents with ethanol above 50% and below 20% and times higher or lower 60 minutes significantly worsen the efficiency of extraction due to lower diffusion rate and solubility of compounds in solvents. Also, lower bioactive yield (about -75%) was observed in all samples extracted by UAE. Previous studies indicated that use of frequencies > 40 kHz during UAE can favour the occurrence of inertial

cavitation phenomena leading to the formation of free radicals and a consequent decrease in substances subject to oxidation (Masuda *et al.*, 2015). Extraction tests revealed that solvents play a key role in the recovery of bioactive compounds. Among the various tested solvents, only the 20, 30 and 50% hydroalcoholic mixtures favoured the recovery of compounds with a more marked antioxidant activity. The lower qualitative yield obtained with the other tested solvents could be caused by the presence in CS of different compounds with different chemical structure and polarity whose recovery is strongly influenced by the affinity with the composition of the mixture (Murthy *et al.*, 2012).

3.2 Physical-chemical and sensory characteristic of gummy candies

The obtained results (Table 1) suggested that the TPC and antioxidant activity were dose-dependent because a significant increase was found after the addition with increasing percentages of CSE. In fact, after the formulation, CS1, CS2 and CS3 showed TPC (273-317 µg GAE g^{-1}) and antioxidant activity (39-46 µmol Trolox g^{-1}) significantly higher than CTR (265 µg GAE g^{-1} and 24.43 µmol Trolox g^{-1}, respectively) and this trend was generally maintained during the storage. The observed results of antioxidant activity despite the evident reduction of TPC during time (about 50%) reveal the possible presence of other chemical compounds with radical scavenging properties, such as melanoidins generated during the coffee roasting process, as also confirmed by previous studies (Tores de la Cruz *et al.*, 2019). The addition of CSE has also affected the variation of other physical and chemical parameters (water activity, solid soluble content, moisture, pH). However, previous studies have reported that maintaining a_w between 0.55 and 0.75 and moisture between 8% and 22% allow to preserve the quality characteristics of the gummy candies over time (Ergun *et al*., 2010). Our observed results denoted a conformity in these ranges. About other physical parameters, increasing concentration of CSE has significantly affected the colour of the candies, resulting in a gradual change in colour from yellow-orange to orange-brown (data not shown) and the mechanical properties, with gradual increase of gumminess. Nevertheless, the results obtained from the sensory analysis indicated that the proposed products were appreciated for all the sensory attributes tested (appearance, olfactory, taste and texture sensations). Specifically, CS1 obtained a higher score (7.7) than the other ones (6.2-7.2) correlated with a greater perception of the fruity. Finally, a microbial quality was confirmed by the absence of moulds and yeasts contamination in all tested enriched gummy candies. The results up to 120 days of storage revealed the higher quality in CS1 gummy candies not only for their bioactive content and antioxidant activity but also for their sensory and structural characteristics. These findings suggested that coffee silverskin can be used as ingredient for preserving the confectionery product quality and increasing their functional properties.

Table 1 *Qualitative parameters of fruit gummy candy samples during the storage.*

Parameters	Days	CTRL	CS 1	CS 2	CS 3	Sign
TPC (µg GAE g^{-1})	0	265.1 ^{c}	272.7 ^{bc}	282.3 ^{b}	317.1 ^{a}	**
	120	131.4 ^{bc}	161.1 ^{a}	128.0 ^{c}	147.9 ^{ab}	**
ABTS assay (µmol Trolox g^{-1})	0	24.43 ^{c}	38.65 ^{b}	42.81 ^{a}	45.75 ^{a}	**
	120	15.06 ^{b}	31.49 ^{a}	30.25 ^{a}	28.20 ^{a}	**
Moisture (g/100 g)	0	19.87 ^{a}	19.97 ^{a}	18.5 ^{b}	18.3 ^{b}	**
	120	17.8 ^{a}	16.8 ^{b}	16.5 ^{b}	13.5 ^{c}	**
aw	0	0.65 ^{b}	0.66 ^{a}	0.65 ^{c}	0.60 ^{d}	**
	120	0.58 ^{c}	0.55 ^{d}	0.64 ^{a}	0.60 ^{b}	**
pH	0	3.86 ^{b}	3.85 ^{b}	3.93 ^{a}	3.94 ^{a}	**
	120	3.90 ^{c}	3.90 ^{c}	4.00 ^{b}	3.99 ^{a}	**
Gumminess (N)	0	9.44 ^{c}	9.66 ^{c}	12.88 ^{a}	11.22 ^{b}	**
	120	61.37 ^{b}	63.58 ^{b}	68.20 ^{ab}	75.83 ^{a}	**
Total acceptability	0	7.2^{b}	7.7^{a}	7.2^{b}	6.8 ^{c}	**
	120	6.5^{b}	7.3^{a}	6.3^{b}	6.0 ^{c}	**

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Biobased approaches at modulating the interaction between legume biopolymers and bioactives: a perspective for the production of baked goods

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The purpose of the second-year activity was to evaluate the impact of different sprouting times (24, 48, and 72 hours) on the biomolecular profile of bean (Vigna unguiculata) seeds. The results show that germination is able to reduce nutritional factors and increase the degree of protein hydrolysis and polyphenols with a maximum effect at 72 hours of germination. Based on the results obtained bakery products (bread) enriched with 72h sprouted bean flour and their respective controls were manufactured, biomolecularly characterized and in vitro digested.

Approcci biomolecolari per lo studio delle interazioni tra biopolimeri e bioattivi nei legumi: una prospettiva futura per la produzione di prodotti da forno

Scopo dell'attività di questo secondo anno è stato valutare l'impatto di differenti tempi di germinazione (24, 48, e 72h) sul profilo biomolecolare di semi di fagiolo (Vigna unguiculata). I risultati evidenziano come la germinazione sia in grado di decrescere i fattori nutrizionali e di aumentare l'idrolisi di proteine e di polifenoli con un massimo effetto a 72h di germinazione. Sulla base dei risultati ottenuti, si è proceduto alla realizzazione, alla caratterizzazione biomolecolare ed alla digestione in vitro di prodotti da forno (pane) arricchiti con la farina fagiolo germinata per 72h e dei rispettivi controlli.

1. Introduction

The Ph.D thesis project aims to evaluate the effect, through a biomolecular approach, of innovative and technological processes on cowpea seeds for the production of bakery products with high nutritional value.
In accordance with the previously described PhD thesis project, the main activities during this second year were:

(A1) A complete evaluation, through a biomolecular approach, of the impact of sprouting bean (*Vigna unguiculata*) seeds conducted for various time (24, 48, and 72 hours) on the presence of anti-nutritional factors such as trypsin inhibitory activity and phytate concentration, oligosaccharide, free polyphenol content, and protein profile.

(A2) Making wheat bread enriched with 25% flour from been seeds sprouted for 72h. The sprouting time was chosen based on the results obtained in previous activities. The baked goods were characterized from a biomolecular point of view (protein profile and anti-nutritional factors) and will be subjected to *in vitro* digestion in order to assess the impact of the functional ingredient on the bioaccessibility of nutrients and bioactive compounds. The results so obtained were compared with the data provided from wheat bread and bread enriched with 25% non-sprouted bean seed flour.

2. Materials and Methods

Seeds were soaked in water (1:3, w/w) for 16 h at 27°C and sprouted in a lab-scale climate chamber for 24h, 48 h and 72 h at 27°C and 90% relative humidity. After sprouting, seeds were dried at 50°C for 8 h and unsprouted seeds were used as the control. All samples were milled into powder in a laboratory mill. In bean powder, protein profile and hydrolysis degree were evaluated by SDS-PAGE in the presence/absence of disulfide reducing agent and OPA assay, respectively. Trypsin inhibitory activity and free phenols content were measured according EN ISO 14902 standard and Folin-Ciocalteau assay, respectively, while phytates and oligosaccharides content by commercial kit following the manufacturer's instructions.
Experimental bread has been prepared according to a traditional recipe (Bresciani A. et al., 2019). Samples bread were: 100% wheat flour bread; wheat bread enriched with 25% of unsprouted bean seed flour; wheat bread with 25% of bean seed 72h sprouted flour. Protein profile and anti-nutritional factors in bread samples were evaluated as described previously. Bread samples will be subjected to in vitro static gastrointestinal digestion according to the INFOGEST protocol, and the kinetics of protein release and bioactive compounds will be detected by sampling at the end of the gastric phase, as well as in the middle and at the end of the intestinal phase.

3. Results and Discussion

The results on the germinated flour indicated that sprouting led to a progressive decrease in antinutritional factors and oligosaccharides, along with an increase in free polyphenols content and protein hydrolysis. The molecular investigation of germinated flour included the detection of -SH group exposure and tryptophan fluorescence by front face fluorescence in order to describe proteins structural features modifications induced by sprouting (Bonomi et al.,2004). The collected data indicated no significant difference in the number of SH group exposed between the germinated and untreated flour samples suggesting no major modification in the overall compactness of protein organization. Conversely, the front face fluorescence spectra (intrinsic tryptophan fluorescence) indicated that overall protein structural modifications were modified by sprouting. All together the data provided from this study indicated that 72 hours of sprouting represents the optimal time, and we used this time to produced bread enriched with sprouted been seed flour. The modifications induced by sprouting in micro/macromolecules profile are maintained in bread suggesting that they were not affected by technological processes. In figure 1 the the proteins profile of the bread sample CB, BB0 and BB72 in reducing (A) and non-reducing conditions (B) are shown. In this figure, protein profiles of the samples, under reducing and non-reducing conditions differ significantly. Sprouting was accompanied by a time-dependent proteolysis of the large soluble aggregates evident under non reducing conditions to produce species of molecular weight around 40 kDa along with smaller peptides that may have escaped detection (Borgonovi SM. et al., 2022).

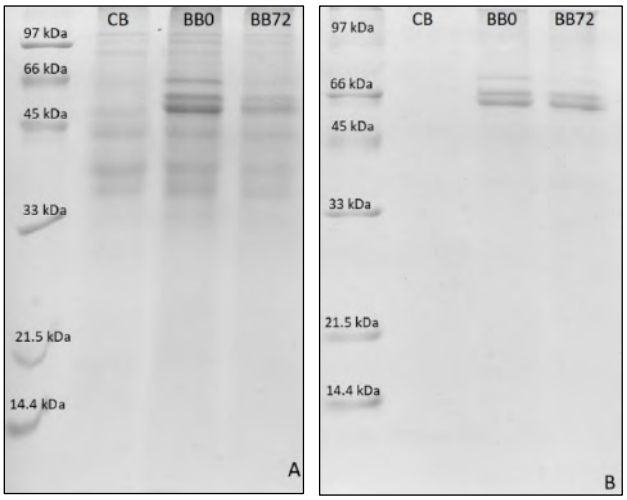

Figure 1 *SDS-PAGE of proteins aqueous extract in the presence (A) and in the absence (B) of reducing agent from wheat- bread (CB) bread enriched with 25% of unsprouted bean seeds flour (BB0), and bread enriched with 25% of 72h sprouted bean seeds flour (BB72).*

The data on micromolecules characterization indicated that bread made with the 25% germinated bean seeds flour show the lowest levels of trypsin inhibitors and phytates when compared to the wheat bread and non-germinated bean seeds flour bread. Further investigations on phenolic and proteins profile of various bread samples before and after in vitro digestion will be carried out during the period abroad at the University of Granada, Spain.
Based on these results, it can be assumed that incorporating sprouted beans flour as a food ingredient may be used for production of bread with an increase in nutritional properties.

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Iron biofortification of oats (*Avena sativa cv. "Prevision"*) grown under aeroponic conditions

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The present contribution reports the results regarding the biofortification of oats aimed at increasing the final iron content of the whole plant. This plant, as many other monocotyledonous plants, absorbs iron through root-exudated compounds known as phytosiderophores. To enhance the iron absorption, plants were grown in iron-free nutritive solution and iron was later added as inorganic iron (iron (III) ammonium sulfate). This treatment was compared with standard nutrition with chelated iron (Fe-EDDHA). Different times of growth without iron and different harvesting post-iron addition were also investigated.

Biofortificazione del ferro in avena (*Avena sativa cv. "Prevision"*) cresciuta in coltura aeroponica

Il presente contributo riporta i risultati riguardanti la biofortificazione dell'avena col fine ultimo di aumentare il contenuto totale di ferro nella pianta. Questa pianta, come molte altri monocotiledoni, assorbe il ferro tramite dei composti essudati dalle radici e conosciuti come fitosiderofori. Al fine di aumentare l'assorbimento di ferro, le piante sono lasciate crescere in soluzione nutritiva priva di ferro e quest'ultimo è aggiunto in un secondo momento sotto forma in ferro inorganico (ferro (III) ammonio solfato). Questo trattamento è quindi confrontato con la crescita standard con ferro chelato (Fe-EDDHA). Sono anche stati investigati tempi diversi di crescita senza ferro e diversi tempi di raccolta.

Key words: Biofortification, iron, oats, phytosiderophores, aeroponic growth.

1. Introduction

The aim of this part of the PhD project is to enhance the nutritional content of an already established microgreen and in particular its iron content. Oat (*Avena sativa* cv. "Prevision") was chosen because its seeds are a well-known source of iron and is also gluten-free, making it suitable as integrator for many kinds of diets. The results of experiments involving iron starvation to force the release of phytosiderophores indicate that this strategy can be successful to biofortify oats. Economical and production advantages are also briefly discussed in view of the shortening of growing/harvesting cycles that was explored in a second series of experiments. Additionally, it is hypothesized that this growing approach can be used for biofortification of other minerals or in co-culturing systems to increase iron content in other iron-inefficient plants.

2. Materials and methods

Seeds of oats (*Avena sativa* cultivar "Prevision") were bought by a commercial seed producer. Plants were grown either at the aeroponic plant of Zero s.r.l. in Pordenone or hydroponically at the lab in Conegliano. "Starvation" experiments were conducted with plants grown for different amounts of time either on a modified Hoagland nutritive solution without iron, the same nutritive solution with iron chelate or simple osmotized water. When inorganic iron was added, it was in the form of iron (III) ammonium sulfate whereas the iron chelate was Fe-EDDHA. Hydroponic growth was performed under comparable parameters, but the nutritive solution was constantly aerated using an aquarium pump and aeration stones.
Plant material was desiccated using a desiccator oven for the time needed to reach constant weight (usually 3-4 days). The desiccated plants were ground to a fine powder using a coffee grinder and mineralized using a combination of sulfuric acid and hydrogen peroxide. The obtained mineralized samples were analysed by AAS.

Table 1: *Avena sativa* "Scura" iron content under different iron fertilization

Treatment type	Iron content (mg/100g DW)
7 days no iron	10,6
+ 5 days 1,2 mg/L Fe-HBED	13,6
7 days 1,2 mg/L Fe-HBED	11,3
+ 5 days 1,2 mg/L Fe-HBED	16,7
8 days 1,2 mg/L Fe-DTPA	14,2

Table 2: *Avena sativa* cv. "Prevision" iron content under different growing conditions. Iron was supplied as inorganic iron at 11 mg/L

Treatment type	Iron content (mg/100g DW)
7 days iron-free nutritive solution	
1 day after iron addition	15,9
2 days after iron addition	23,8
4 days after iron addition	37,2
7 days osmotized water	
1 day after iron addition	38,4
2 days after iron addition	43,0
4 days after iron addition	60,9

3. Results and discussion

Exploratory experiments have demonstrated that the addition of iron in the form of iron chelate (Fe-HBED and Fe-DTPA) has little to no effect on the amount of iron found in the plant (Table 1). These results are in accordance with the literature (Jolley and Brown, 1989b), which shows that oat uptake of iron in the form of chelates is less efficient. Similarly, it was noted that when plants were grown under iron starvation they would show a high uptake capacity from inorganic sources of iron (Reid et al., 1989).

Following the experimental setup of Reid and colleagues, oats were grown for 7 days in osmotized water or nutritive solution without iron. After this time iron was added as inorganic iron. Plants starved in this manner showed a marked increase in iron content (Table 2).
In another set of experiments, plants were grown in standard nutritive solution (containing Fe-EDDHA), iron-free nutritive solution or osmotized water. A longer timeframe was allowed to pass before iron addition and post-addition harvesting was also delayed longer. Also in this case plants starved for iron showed a much higher iron content. Additionally the experiment confirmed that iron nutrition through chelates is ineffective (Table 3).

These results set a promising framework for the efficient and minimally invasive biofortification of oat microgreens. Varying the timeframe has proven that shorter cycles are effective as much as longer ones. Shortening the growing and harvesting periods leads to lower expenditure for lighting, lower risk of contamination during growth and higher number of growing cycles per year. Furthermore, increasing the amount of inorganic iron to 22 mg/L didn't cause any appreciable difference from the fertilization at 11 mg/L, allowing a reduction of inorganic fertilizer needed.

Table 3: *Avena sativa* cv. "Prevision" iron content under different growing conditions. Iron was supplied as Fe-EDDHA at 1,4 mg/Lor as inorganic iron at 22 mg/L

Treatment type	Iron content (mg/100g DW)
10 days standard nutritive solution	
4 days after	6,9
10 days after	7,3
10 days iron-free nutritive solution	
4 days after iron addition	16,0
10 days after iron addition	21,1
10 days osmotized water	
4 days after iron addition	64,7
10 days after iron addition	62,4

Since phytosiderophores have varying affinities for several other elements (calcium, zinc, magnesium etc.) it is of further interest to investigate if the "starvation" setup can be utilized to force the uptake of other minerals of interest. Another path for biofortification is the co-culturing of crop plants, which has been already tested in hydroponic systems (Cesco et al., 2006). In this case oat plants are grown together with other iron-inefficient plants to enhance the iron uptake of the latter plants. Alternatively, a phytosiderophore-rich nutritive solution can be used as a base for growing iron-inefficient plants, constituting a form of chelated iron fertilizer that is totally of plant origin.

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Assessment of the Stability and Efficacy of a Newly Developed Probiotic Blend in the Context of IBS through a Pilot Multicentre Study

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The first two activities of the PhD thesis project are described. Firstly, the adhesion and antioxidant abilities of bacteria that composed the probiotic blend were assessed. These are two of the main probiotic properties evaluated to characterize the bacterial strains from the functional point of view *in vitro*. Secondly, the effect of the probiotic multi-strains supplement on non-constipated IBS patients was assessed, and the results obtained from the microbiomic and metabolite analysis of the faecal samples collected during the clinical study are described.

Valutazione della stabilità e dell'efficacia di un nuovo prodotto probiotico multi-ceppo nel contesto dell'IBS tramite uno studio pilota multicentrico

Sono descritte le prime due attività del progetto di tesi di dottorato. Sono state determinate la capacità adesiva e antiossidante dei batteri contenuti nel blend probiotico, le quali rappresentano due delle principali proprietà utilizzate per caratterizzare *in vitro* la funzionalità dei ceppi. Si è poi valutato l'effetto del probiotico multi-ceppo in soggetti con IBS non costipati, descrivendo i risultati ottenuti dall'analisi microbiomica e dei metaboliti a partire dai campioni fecali raccolti nello studio clinico.

1. Introduction

In accordance with the PhD project, this poster reports the main results of the first 2 activities, which are as follows:

(A1) *in vitro* characterization of strains' probiotic properties from the functional perspective, particularly by evaluating adhesion and antioxidant abilities of bacterial strains;

(A2) assessment of the effect of the probiotic multi-strain supplement in non-constipated IBS patients, with the primary endpoint being the modulation of the faecal bacterial community structure and metabolites.

2. Materials and Methods

Bacterial adhesion to Caco-2 cell line: 2.0×10^8 bacteria for each strain were incubated for 1 h at 37°C with a fully differentiated monolayer, then washed three times with PBS and incubated with 3 ml of methanol for 8 min. Afterwards, cells were stained with 3 mL of Giemsa solution (1:20) and left 30 min at room temperature in the dark. Finally, monolayers were washed and examined microscopically.

Evaluation of antioxidant activity of probiotic bacteria: free radical scavenging activity of strains was measured by mixing 500 µl of each bacterial cell concentration (1.0×10^{10}, 5.0×10^9, 2.5×10^9, 1.0×10^9 cells/mL) with 500 µl of 0.4 mM DPPH-ethanol solution. The control group included 0.1 M phosphate buffer and DPPH-ethanol solution, while blank group contained sample and ethanol. The mixtures were incubated at 37°C in the dark for 30 min, then the optical absorbance was measured at 517 nm after samples centrifugation at 10000 × *g* for 3 min.

Assessment of the cellular antioxidant activity in Caco-2 cells: after 15 days of growth, Caco-2 seeded at 1.0×10^4 cells/well on a black 96-well microplate were treated with 100 µl of 10 µM DCFH-DA up to 30 min at 37°C. Subsequently, the cells were washed with PBS and treated with 100 µl of 0.6 mM ABAP together with bacterial suspensions at MOI of 50, 100, 200 and fluorescence was measured for 13 cycles at 5-min intervals (λ excitation = 485 nm and λ emission = 538 nm). N-acetyl cysteine was used as positive control.

Faecal microbiome analysis: the bacterial community structure of faecal samples was studied by 16S rRNA gene profiling with Illumina HiSeq technology. After the extraction of the total DNA from 150 mg of faeces, the 16S rRNA gene amplicons encompassing the V3 and V4 variable regions were sequenced. Moreover, the concentration of short-chain fatty acids (SCFAs; acetate, butyrate, propionate, valerate, isovalerate) and organic acids (lactate and succinate) was quantified in faecal samples by UPLC-MS.

3. Results and Discussion

3.1 Definition and microbiological characterization of the probiotic blend

The differentiated Caco-2 epithelial cell layer was used to test the potential ability of the bacterial cells to adhere on human enterocytes. As expected, since *Bifidobacterium bifidum* MIMBb23sg has been previously demonstrated to be strongly adhesive, it resulted with the higher adhesion index (i.e., bacterial cells per 100 Caco-2 cells) compared to the other strains (Table 1), and this may play a pivotal role in increasing the intestinal barrier with a concurrent beneficial effect in IBS patients (Guglielmetti et al., 2011).

Table 1 *Adhesion properties of the probiotic bacterial strains to a Caco-2 cell monolayer. Data are reported as adhesion index.*

Enterolactis® Ultra *strains*	***Adhesion index***
Bifidobacterium bifidum MIMBb23sg (*Bifidobacterium bifidum* BbfIBS01, DSM 32708)	>2000
Lacticaseibacillus paracasei DG I1572; L. casei DG® (DSM 34154)	>50
Bifidobacterium breve BbIBS01 (DSM 33231)	>60
Bifidobacterium breve BbIBS02 (DSM 33232)	>60
Lactiplantibacillus plantarum LpIBS01 (DSM 33234)	>100
Bifidobacterium animalis subsp. *lactis* BlIBS01 (DSM 33233)	>100

The DPPH assay was used as a screening for assessing strains' antioxidant abilities and was defined as scavenging activity, while the cellular antioxidant assays was used to reveal the total antioxidative capacity of the strains by measuring ROS accumulation in Caco-2 cells. The ROS scavenging abilities were consistent with the CAA results, showing that both MIMBb23sg and DG exert a statistically significant ability to lower ROS in a dose-dependent manner. The free radical scavenging ability is plausibly due to the properties of the molecules on the bacterial cell outer surface (e.g., the exopolysaccharide of strain DG; Balzaretti et al., 2017). Reportedly, some probiotic strains are reported to scavenge oxygen free radicals protecting Caco-2 cells against damage, maintaining Caco-2 cell integrity by enhancing the expression of tight junction proteins to protect the host against ROS injury (Mu et al., 2019).

3.2 *In vivo* clinical trial

Regarding the effect of the probiotic treatment on faecal taxonomic diversity, α-diversity was not significantly changed. Furthermore, the analysis of inter-sample biodiversity (β-diversity) indicated that the treatments did not induce a significant alteration in the overall bacterial community structure of faecal samples. However, concerning the impact of the probiotic intervention on specific faecal bacterial taxa, the administration of probiotics resulted in a significant decrease of the phylum *Actinobacteria* and a significant increase of the phylum *Bacteroidetes*. In addition, several taxa of the phylum *Proteobacteria*, such as the genera *Paracoccus*, *Ralstonia*, *Halomonas* and *Vibrio*, were significantly reduced after probiotic treatment compared to the placebo.

Therefore, the daily intake of a sachet of Enterolactis® Ultra modifies the intestinal microbial ecosystem of non-constipated IBS patients. It also led to a significant reduction in the ratio between the faecal levels of the SCFAs propionate and butyrate, as reported in Table 2. Notably, the propionate:butyrate ratio has already been proposed as a potential biomarker for IBS in previous studies, showing that the difference between propionic acid and butyric acid (mmol/l) was significantly higher in diarrhoea-predominant IBS patients (Farup et al., 2016).

Finally, correlation analysis revealed significant positive associations of propionate and the propionate/butyrate ratio with several bacterial taxa that resulted significantly reduced by the probiotic intervention, including the genus *Collinsella*, the family *Leuconostocaceae* and the genus *Coprobacillus*, which resulted also showed negative correlation with butyrate levels.

Table 2 *Faecal concentration of organic acids in patients participating to the clinical trial. Data are presented as median values and expressed as mmol/100 g of faeces. *, P<0.05 based on non-parametric ANOVA.*

		P	**Before Probiotic**	**After Probiotic**	**Before Placebo**	**After Placebo**
Acetate		0.537	3.66	4.21	4.03	3.93
Butyrate		0.152	2.23	2.95	3.98	2.55
Propionate		0.100	1.08	0.97	0.92	1.15
Valerate		0.140	1.24	1.13	1.21	1.42
Succinate		0.555	0.09	0.15	0.10	0.09
Isovalerate		0.478	0.56	0.59	0.57	0.53
Lactate		0.355	0.04	0.05	0.03	0.03
Acetate/Butyrate		0.468	1.23	1.25	1.21	1.29
Propionate/Butyrate	*	**0.013**	**0.42**	**0.22**	**0.26**	**0.38**

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Valorisation of Myrtle Liqueur Processing and Olive Pomace By-Products as Ingredients for Functional Dairy Foods

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The aim of this PhD project is to study the valorisation of myrtle liqueur processing (MLP) and olive pomace (OP) by-products as ingredients to produce a functional yogurt made from whole ovine milk. Yogurts were produced with different processes and analyzed from the chemical-physical and rheological point of view. The final goal of this thesis will be both to optimize the process conditions to produce fortified yogurts, through the choice of the best chemical, rheological, and sensory properties, and to evaluate the potential human benefits through the evaluation of their fiber content and antioxidant activity.

Valorizzazione dei sottoprodotti della lavorazione del liquore di mirto e della sansa di oliva come ingredienti per alimenti lattiero-caseari funzionali

L'obiettivo di questo progetto di dottorato riguarda la valorizzazione dei sottoprodotti della lavorazione del liquore di mirto e della sansa di oliva come ingredienti per la produzione di uno yogurt funzionale a base di latte ovino intero. Gli yogurt sono stati prodotti con due processi tecnologici differenti e analizzati dal punto di vista chimico-fisico e reologico. L'obiettivo finale sarà ottimizzare le condizioni di processo per la produzione di yogurt fortificati, attraverso la scelta delle migliori proprietà chimiche, reologiche e sensoriali, e valutare i potenziali benefici per la salute umana attraverso la determinazione del loro contenuto in fibre e dell'attività antiossidante.

Key words: By products, whole ovine milk, fortified yogurt, rheology, texture, total phenolic content.

1. Introduction

Globally, according to the FAO reports (2011), one-third of food production for human consumption (1.3 million tons) is lost or wasted along the food chain, from primary production to the final consumer. This involves both inedible (pits, pomace, peels, etc.) and edible components (fruit and vegetable peels, unripe or damaged fruits and vegetables) (Trigo et al., 2019). In Italy, waste generation from the food and beverage industry has been estimated at about 3.4 million tons in 2019. There is a need to shift from a linear economy, which involves waste generation, to a circular economy that aims to extend the life cycle of products while minimizing waste generation. According to this, the research project focuses on:

(A1) the valorisation of two important by-products of the Sardinian food supply chain, which have been added in whole ovine milk;
(A2) to produce fortified yogurts enriched in polyphenols and antioxidant activity.

2. Materials and Methods

Fat, protein, lactose and casein of sheep's milk used for yogurt production were analyzed using the FTIR method (Milkoscan FT + Foss Electric, Hillerød, Denmark). Yogurts produced were fortified with 1% (w/w) of dried MLP or freeze-dried OP by-products following this experimental design: CTRL, whole ovine milk without fortification; Treatment, obtained fortifying the milk before heat treatment; Broken Treatment, obtained fortifying the finished yogurt 24h after production during the clot-breaking and yogurt-potting phase. The pH of yogurts was measured with a pH-meter at constant intervals until reaching the pH of 4.67. A penetration test was carried out on yogurt samples using a TA.XT plus texture analyzer (Stable Micro System, UK) equipped with a P-25 probe and 5 kg load cell to measure the firmness, work of shear, stickiness and work of adhesion. Steady shear and frequency sweep test were also performed by using a rheometer (MCR-92, Anton Paar, Graz, Austria) with a plate to plate system (diameter:50 mm, gap: 0.5 mm) at 25°C. With the steady shear analysis performed at a shear rate of 1-1000 s^{-1} the consistency index and apparent viscosity at 50 s^{-1} were determined. For the frequency sweep, the viscoelastic properties of each yogurt sample were obtained by analysing the dynamic shear rheological properties (G'; G''; tan δ= G''/G'). In addition, the total phenolic content (TPC) was determined following the method of Noriega-Rodriguez et al. (2020) with modifications. Finally, water holding capacity (WHC) was determined by centrifuging at 2500 rpm, 4°C, 25 g of yogurt sample for 20 min (Osorio-Arias J., et al., 2020) and syneresis by the drainage method (Amatayakul et al., 2006).

3. Results and Discussion

3.1. Ovine milk composition

The composition of ovine milk used to produce fortified yogurts with OP (mean of four batches) and MLP (mean of four batches) was as follows: fat, 5.68 ± 0.11% (w/w); protein, 4.74 ± 0.2% (w/w); fat/protein ratio,1.20 ± 0.02; lactose, 4.61 ± 0.12% (w/w); casein, 3.52 ± 0.05% (w/w) for OP and fat, 4.45 ± 0.30% (w/w); protein, 4.86 ± 0.16% (w/w); fat/protein ratio, 0.92 ± 0.08; lactose, 4.59 ± 0.19% (w/w); casein, 3.63 ± 0.15% (w/w) for MLP. The eight batches were obtained between december 2022 and may 2023.

3.2 Acidification curve

Based on the trend of the acidification curves, the addition of OP in yogurts before the heat treatment, decreases the time to reach pH 4.6 from 5 h 24 min to 5 h (Fig. 1a); in contrast, the addition of MLP in yogurts increases the time to reach pH 4.6 from 5 h 20 min to 6 h 20 min (Fig. 1b). It can be supposed that the polyphenols present in the two classes of by-products (OP, MLP) could promote or slow down the acidification process.

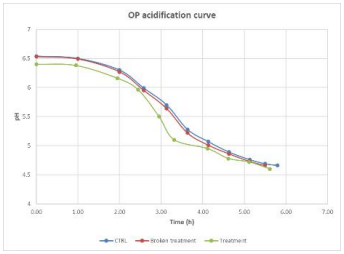

Fig.1a

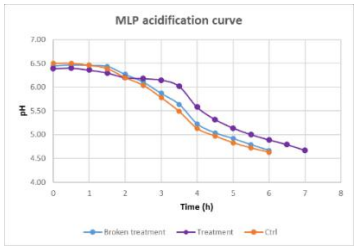

Fig. 1b

3.3 Texture analysis

Penetration data were reported in the *Table 1*. For OP yogurt samples a tendency to be firmer and stickier than control was observed for the Treatment but only the Broken Treatment was significantly less firm and sticky than the Treatment and the CTRL, while for MLP yogurt samples, both the Treatment and the Broken Treatment resulted significantly less firm and sticky respect to the CTRL.

Table 1 *Texture properties of fortified yogurts.*

Sample	Firmness (+1% OP) N Force 1	Stickiness (+1% OP) N Force 2	Firmness (+1% MLP) N Force 1	Stickiness (+1% MLP) N Force 2
CTRL	0.43 ± 0.03^{a}	-0.15 ± 0.02^{b}	0.47 ± 0.06^{a}	-0.16 ± 0.03^{a}
TREATMENT	0.44 ± 0.04^{a}	-0.16 ± 0.02^{b}	0.35 ± 0.08^{c}	-0.11 ± 0.05^{b}
BROKEN TREATMENT	0.39 ± 0.03^{b}	-0.12 ± 0.02^{a}	0.41 ± 0.06^{b}	-0.14 ± 0.03^{b}

3.4 Rheological analysis: Steady shear and Frequency sweep

Data of steady shear analysis confirmed what stated above, observing a slight increase of the consistency index for yogurt fortified with 1% OP (Treatment), whereas a significant reduction was observed in yogurt fortified with 1% MLP (Treatment). With reference to the Frequency sweep analysis no significant difference in Tan δ values was observed for OP fortified yogurts. On the contrary, a significant increase was found in Tan δ values for MLP Treatment and Broken Treatment, indicating a decrease of the elastic behaviour respect to the viscous. Overall, all yogurt samples exhibited solid-like behaviours and weak elastic gel structures.

3.5 Total phenolic content, syneresis and water holding capacity

Total phenolic content (TPC) was significantly higher for OP Treatment and OP Broken Treatment respect to the CTRL. The OP Treatment also exhibited an higher water-holding capacity and lower syneresis than CTRL and Broken Treatment. In the MLP yogurts the Treatment showed the highest TPC value, while no significant differences in syneresis and WHC were found among the three samples (*Table 2*).

Table 2 *Total phenolic content, syneresis and WHC of fortified yogurts*

Sample	**TPC** (mg/100g sample) (+1% OP)	**Syneresis** (%) (+1% OP)	**WHC** (%) (+1% OP)	**TPC** (mg/100g sample) (+1% MLP)	**Syneresis** (%) (+1% MLP)	**WHC** (%) (+1% MLP)
CTRL	2.32 ± 0.29^{b}	11.32 ± 2.30^{a}	84.02 ± 3.11^{b}	2.16 ± 0.36^{c}	11.78 ± 1.84^{ns}	80.75 ± 3.49^{ns}
TREATMENT	14.70 ± 3.48^{a}	5.27 ± 1.21^{c}	92.21 ± 1.76^{a}	9.40 ± 0.40^{a}	12.40 ± 4.92^{ns}	78.13 ± 6.48^{ns}
BROKEN TREATMENT	13.83 ± 4.23^{a}	9.12 ± 1.57^{b}	82.76 ± 3.33^{b}	7.56 ± 0.56^{b}	10.54 ± 2.85^{ns}	76.02 ± 3.60^{ns}

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Eco-design tools development for sustainability optimization in food production systems

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This PhD project aims to develop eco-design tools based on Life Cycle Assessment (LCA) methodology to provide food systems with a reliable resource when moving towards sustainability. The project aims to identify the most appropriate models to accurately assess the eco-profile of selected food products, processes or systems and to provide a user-friendly and strategic decision-making tool for businesses.

Sviluppo di strumenti di eco-design per l'ottimizzazione della sostenibilità nei sistemi di produzione alimentare

Questo progetto di dottorato mira a sviluppare strumenti di eco-design basati sulla metodologia Life Cycle Assessment (LCA) per fornire ai sistemi alimentari una risorsa affidabile nel passaggio verso la sostenibilità. Il progetto mira a identificare i modelli più appropriati per valutare accuratamente l'eco-profilo di prodotti, processi e sistemi alimentari e fornire uno strumento decisionale strategico e di facile utilizzo per le imprese.

Key words: Eco-design, Life Cycle Assessment (LCA), sustainability, eco-profile, decision-making tool.

1. Introduction

The agri-food system has been identified as a significant contributor to environmental impacts, as highlighted in the United Nations Sustainable Development Report (2019). In recent years, there has been a notable increase in the importance of environmental sustainability as a critical factor affecting business performance (Bernal Torres et al., 2021; Adams et al., 2021). Both policymakers and consumers are putting increasing pressure on companies to prioritise environmental friendliness, not only within their internal processes but also across their entire value chain, including customers and suppliers (Mata et al., 2012). Environmental sustainability goals often influence product development and business decisions, but the production process is rarely optimised to address these concerns (Linnemann et al., 2006). To answer this need, different solutions based on the Life Cycle Management (LCM) method to guide companies in measuring sustainability are increasing.

2. Materials and Methods

As a first part of this PhD project, a systematic review (Casson et al., 2023) was conducted to assess the available simplified tools in the agri-food sector. The aim of the review was to provide a comprehensive analysis of environmental sustainability tools used in the agri-food chain for both academic and business communities. The focus was on simplified environmental impact tools and calculators applicable to agriculture, food processing and the wider agri-food system. The review identified quality parameters and carried out the multivariate analysis, which allowed differentiation between the 79 tools examined.
Based on the findings of the review, an eco-design tool was developed in collaboration with the research and development team of a nutraceutical company based in Lombardy (Italy). The development of all data sets started with a study of all raw materials used by the company, reviewing all production flowsheets and raw material emission models for all raw materials and the associated direct emissions from ingredient formulation. The obtained datasets were then incorporated into the tool as a library source of primary data. The tool has been developed using a cradle-to-gate approach, which allows the life cycle of the intended product (1 package of the finished product) to be studied, from the extraction of raw materials to the finished product being packaged and ready for shipment.

3. Results and Discussion

3.1 Systematic review findings

The systematic review results highlighted a clear separation between the simpler tools, characterised by high usability, low data entry requirements and accessibility, and the more complex tools, characterised by transparency and presentation of results.

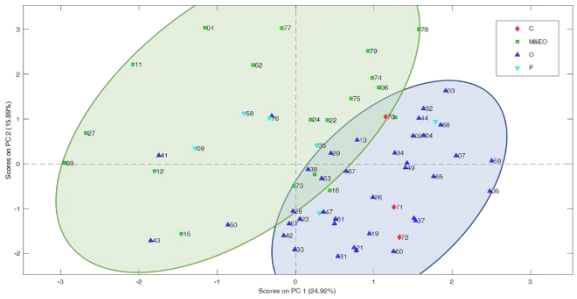

Figure 1 *Scores plot highlighting tools grouped by potential users*.

A biplot analysis was performed to assess the significance of the different factors among the analysed tools, taking into account their multivariate nature. Figure 2 shows the PCA biplot generated from the same data set as the score plot in Figure 1. The biplot shows the samples coloured according to their intended user categories, including consumers, operators, managers, environmental offices and policymakers. It highlights the key quality parameters that differentiate tools based on their intended users. Tools designed for simplicity (shown as blue triangles) have high usability, low data entry requirements and are accessible to a wider range of users. Notably, transparency and accessibility are positioned opposite each other at the intersection of the axes, representing the characteristics of tools intended for more experienced users, located in the upper left-hand section with negative values of PC1 and positive values of PC2.

PCA was used to analyse the influence of quality parameters on simplified environmental impact tools and to generate a scores plot representing the current scenario. The plot showed two clusters: tools for operators and consumers, represented by blue triangles and red diamonds, were located within the blue circle, while tools for managers and policymakers, represented by green squares and light blue triangles, were located further away within the green circle. Although the first two components (PC1 and PC2) explained about 40% of the total variability, the different clusters indicated different user requirements and levels of complexity within the simplified environmental impact tools tool system.

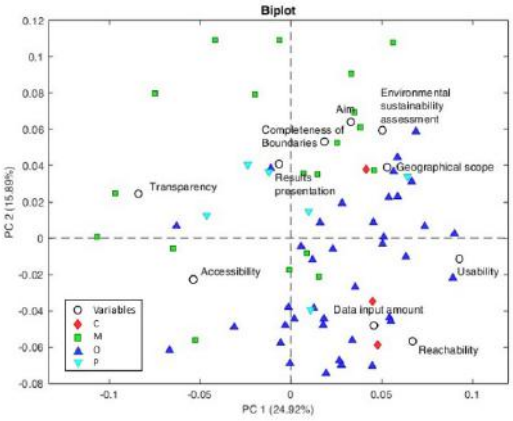

Figure 2 *Biplot deriving from PCA, showing tools and quality parameters.*

3.2 Development of LCA tool for Ecodesign

Based on the results of the review, a TaylorMade environmental impact tool, called MAPPER, has been developed following the reference Product Category Rules (PCR) guidelines. The LCA model was built without the approximations typical of the simplified eco-design tools, allowing for more accurate and certifiable environmental footprints of products without significant changes to the calculation model. The obtained tool incorporates convenient accessibility, achieves the organisational objective and demonstrates relevance to the intended application. It has minimal data entry requirements and uses a transparent computational framework based on recognised patterns that suggest different impact categories. The MAPPER can help to reduce the time and effort required to perform environmental assessments, allowing Research and Development department, designers, and engineers to quickly evaluate different ingredient options and identify areas for improvement. This can lead to more efficient and sustainable product choices, ultimately reducing the environmental footprint of products and processes.

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Effect of fermentation of selected lactic acid bacteria on the technological properties of sorghum-composite bread

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The first 2 activities of the Ph.D. project are described. The growth capacity of selected lactic acid bacteria on sorghum and their effect on the thermal, rheological, molecular, antioxidant, and color properties of sorghum flour were evaluated. The effect of fermentation on the technological properties of sorghum-composite bread was then evaluated in terms of specific volume, texture, a_w, moisture content, color, and sensory acceptability.

Effetto della fermentazione da parte di batteri lattici selezionati sulle proprietà tecnologiche di pane composito di sorgo

Vengono descritte le prime 2 attività del progetto di tesi di dottorato. È stata misurata la capacità di crescita di batteri lattici selezionati sulla farina di sorgo e il loro effetto sulle proprietà termiche, reologiche, di mobilità molecolare, di colore e di capacità antiossidante, sulla farina di sorgo. È stato poi valutato l'effetto dal punto di vista tecnologico della fermentazione della farina di sorgo sul pane composito, valutando il volume specifico, la consistenza, a_w, il contenuto di acqua, il colore, e l'accettabilità sensoriale.

Keywords: sorghum, bread, functional properties, LAB fermentation.

1. Introduction

Following the previously described Ph.D. project (Chiodetti, 2022), this poster reports the main results of the first two activities concerning:

(A1) Selection of functional LAB strains and subsequent fermentation of sorghum flour. Determination of the effect of the LAB activity on the techno-functional properties sorghum flour;

(A2) Evaluation of the effect of the addition of sorghum liquid sourdough on the properties of fresh wheat composite bread. Assessment of bread consumer acceptability.

2. Materials and Methods

Lactobacillus delbruekii subsp. *bulgaricus* 1932, *Lacticaseibacillus casei* 4339, and *Leuconostoc* spp. 4454, previously selected according to their proteolytic, aromatic, and EPS-producing activities, were used for the liquid sourdough fermentation of sorghum flour (25°C, 15h). To assess the growth capacity of bacteria on sorghum, the pH, total titratable acidity (TTA), and microbial counts in the sourdoughs were analyzed. Moreover, the color, antioxidant activity (DPPH assay), viscosity (rheometer; 25°C, 0.1-1000 1/s), pasting properties (rheometer; 160 rpm, heating 50-95°C, holding 95°C for 5 min, cooling 95-25°C, 5°C/min), thermal properties (DSC; heating 30-130°C, 5 °C/min), and ^{1}H molecular mobility (Time Domain ^{1}H NMR; self-diffusion coefficient D, CPMG $^1HT_2$) were evaluated in the sourdough samples. To evaluate the effect of fermentation on sorghum-composite bread, wheat bread with 25% sorghum sourdough and a control bread (from wheat and unfermented sorghum flour), were characterized in terms of specific volume, a_w, moisture content, color, texture (TPA, 40% compression, 35 mm cylindrical probe) and antioxidant activity. The consumer sensory acceptability of sorghum-composite breads was evaluated with an acceptability test (n = 58 untrained judges), using a nine-point hedonic scale. Overall, three independent batches were analyzed for each sample. Statistical differences were evaluated with one-way ANOVA and Tukey post hoc test (α=0.05) with SPSS software (v. 27.0, SPSS Inc., Chicago, USA).

3. Results and Discussion

3.1 Effect of fermentation on the functional properties of sorghum flour

All LAB strains showed excellent growth capacity on sorghum, reaching values of up to 10^9 CFU g^{-1}, and pH between ≈ 4.2 and 4.5 (Table 1). Moreover, fermentation increased the total titratable acidity of sourdoughs. After fermentation, the increase in the acidity and consequently a decrease in pH of sourdoughs showed a pH-induced color change, with increased a*, b*, and L* in fermented samples. The changes that occurred in the color properties can be probably related to changes affecting the phenolic compounds (Olojede et al., 2022). The antioxidant activity also increased after fermentation (with the highest value for sample *L.* 4454). This result can be related to the effect of hydrolysis and the release of bound antioxidant compounds during fermentation and *de novo* synthesis of compounds with antioxidant activity (Gobbetti et al., 2019). Furthermore, fermentation increased the viscosity of sourdoughs, especially in the sample *Lcb.* 4339, which showed the highest viscosity. The increase in viscosity

could be due to a hypothetical effect of EPS production and changes in the proteins and starch fractions.
In all samples, fermentation increased the ability of starch to gelatinize, as an increase in the enthalpy of the endothermic peak of starch was detected in DSC. Fermentation by *Lb*. 1932 and *Lcb*. 4339 also increased peak and final viscosity of corresponding sourdoughs. However, the fermented samples showed an increase of the onset temperature of the starch gelatinization and, in *Lcb*. 4339, an increase in the pasting temperatures. The increase in gelatinization temperatures may be related to structural changes in the amylopectin fraction occurring during fermentation (Ye et al., 2019).
The ^{1}H NMR analysis suggested that the highest matrix breakdown occurred in *Lb*. 1932, as highlighted by the highest value of the self-diffusion coefficient ^{1}H D in this sample. The matrix breakdown was also suggested by the higher number of ^{1}H T_2 populations in the fermented samples (four populations: A, B, C, D) compared to the standard (three populations). Among the fermented samples, *Lb.*1932 showed the highest abundance and relaxation time (ms) of Pop D; this population represents the weakly bound OH protons of water. In contrast, the highest abundance of Pop C, related to the exchanging protons of water interacting with starch and proteins, was found in *Lcb*. 4339 (Marchini et al., 2021).

Table 1 *pH of sourdough (SD), bread dough (D), and bread (B) of the experimental samples. STD = unfermented; Lb. 1932 = fermented with Lactobacillus delbruekii subsp. bulgaricus 1932; Lcb 4339 = fermented with Lacticaseibacillus casei 4339; L. 4454 = fermented with Leuconostoc spp. 4454. Different letters indicate significant differences (p≤0.05) between different samples for the same substrate.*

pH substrate	*STD*	*Lb. 1932*	*Lcb. 4339*	*L. 4454*
SD	*6.26±0.01 a*	*4.48±0.04 b*	*4.24±0.02 c*	*4.36±0.09 bc*
D	*5.54±0.02 a*	*5.15±0.03 b*	*5.03±0.01 c*	*5.05±0.01 c*
B	*5.47±<0.01 a*	*5.07±0.02 b*	*4.96±0.04 c*	*5.02±0.01 bc*

3.2 Effect of sorghum fermentation on the bread overall quality

The specific volume of the bread loaves did not change after fermentation, except for sample *Lcb*.4339, in which specific volume resulted slightly but significantly reduced, probably due to the acidic weakening of the gluten network and thus the ability to retain gas during rising and baking (Su et al., 2019) (Figure 1). The fermented loaves also had a higher crumb moisture content. The hypothetical presence of water-binding molecules, such as EPS, could explain the higher crumb moisture in fermented bread, as they could have reduced water loss during baking (Lynch et al., 2018). The color changes for the a* parameter found in the sourdough were also evident in the crumb, which showed a more intense red color compared to the standard. The texture properties improved after fermentation in *Lb*.1932 and *L*. 4454 loaves, showing higher cohesiveness. On the other hand, sample *Lcb* 4339 was harder than the standard. However, regarding the bread sensory acceptability test, there were no significant differences on the texture and color of the loaves, while fermentation improved the overall appearance in sample *Lcb*. 4339. Finally, sample *Lb*. 1932 had the least pleasant odor acceptability. Therefore, these results showed that fermentation can improve some properties of sorghum composite bread. However, more in-depth studies are needed to verify the production of EPS, the role of fermentation in bread staling, and the effect of different methods of addition of sourdough on bread quality, following the original thesis project.

Figure 1 *Experimental bread samples. STD = control bread; Lb. 1932 = bread fermented with Lactobacillus delbruekii subsp. bulgaricus 1932; Lcb 4339 =. bread fermented with Lacticaseibacillus casei 4339; L. 4454 = bread fermented with Leuconostoc spp. 4454.*

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The potentiality of non-*Saccharomyces* yeast derivatives as enological bio-adjuvants

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To date, the yeast derivatives allowed in winemaking are only those obtained from strains belonging to the genus *Saccharomyces* and there are many scientific works that have highlighted their impact on the qualitative characteristics of wine. Conversely, derivatives obtained from non-*Saccharomyces* yeasts have not yet been accepted in winemaking, despite their use as mixed starters for alcoholic fermentation have already been accepted since several years. The advantages deriving from their use in winemaking have been associated not only with their specific metabolism during alcoholic fermentation, but also with the composition of their cell wall, with particular regard to the polysaccharide fraction. Therefore, after having produced some inactivated non-*Saccharomyces* yeast derivatives on a laboratory scale, we proceeded with their evaluation, initially on a model solution and subsequently on a Trebbiano white wine. In particular, their impact on protein stability, colour and aromatic profile was evaluated.

Derivati di lieviti non-*Saccharomyces* come potenziali bio-coadiuvanti enologici

Ad oggi i derivati di lievito ammessi in vinificazione sono solo quelli ottenuti a partire da ceppi appartenenti al genere *Saccharomyces* e molteplici sono i lavori scientifici che hanno evidenziato il loro impatto sulle caratteristiche qualitative del vino. Al contrario, i derivati ottenuti da lieviti non-*Saccharomyces* non sono ancora stati ammessi in vinificazione, nonostante il loro utilizzo come starter misti per la fermentazione alcolica sia stato già ammesso da diversi anni. I vantaggi derivanti dal loro utilizzo in vinificazione sono stati associati non solo al loro specifico metabolismo durante la fermentazione alcolica, ma anche alla composizione della loro parete cellulare, con particolare riguardo alla frazione polisaccaridica. Pertanto, dopo aver prodotto su scala di laboratorio alcuni derivati inattivati di lieviti non-*Saccharomyces*, si è proceduto con la loro valutazione, inizialmente su soluzione modello e successivamente su un vino bianco Trebbiano. In particolare è stato valutato il loro impatto sulla stabilità proteica, sul colore e sul profilo aromatico.

Key words: non-*Saccharomyces yeast*, yeast derivatives, colloidal stability, antioxidant activity, aromatic profile

1. Introduction

In accordance with the PhD thesis project, this poster reports the main results of the first activities concerning:
(A1) characterization of IDYs in wine like solution
(A2) evaluation of IDYs impact on a Trebbiano white wine.

2. Materials and Methods

Saccharomycodes ludwigii (SL) and *Starmerella bacillaris* (SB) represent two of the eight non-*Saccharomyces* strains used as inactivated dry yeasts (IDYs) in this PhD project, and selected for the present report. A commercial strain of *Saccharomyces cerevisiae* (SC) was used as reference strain for *Saccharomyces* and for comparison determination.
For the first activity (A1), IDYs were added onto a wine like solution (ethanol 12% v/v, tartaric acid 4.5 g/L, pH 3.2), and kept in contact for 48 h. After that, polysaccharides quantification (Romani et al, 2020) and evaluation of their molecular weight profiles (Fanzone et al, 2012), were performed. Quantification of GSH (Tirelli et al, 2010) was also determined.
For the second activity (A2), the IDYs were added onto a Trebbiano wine and left in contact for 15 days. After that, the following analyses were performed: quantification of total polysaccharides; evaluation of wine protein stability (by the heat test: 80°C for 2h, 4°C for 16h, RT for 2h) and of colour indexes (by CIELab); quantification of the aroma compounds (by SPE-GC/MS).

3. Results and Discussion

3.1 Characterization of IDYs in wine like solution

In comparison with IDY- SC, IDY-SL and IDY-SB, showed a higher capacity to release polysaccharides in the media (figure 1). These results are likely due to their specific cell wall composition. Moreover, both IDY-SL and IDY-SB showed a higher concentration of polysaccharides with molecular weight > 250 kDa, as compared to those of IDY-SC. Considering that polysaccharides with different molecular weight have

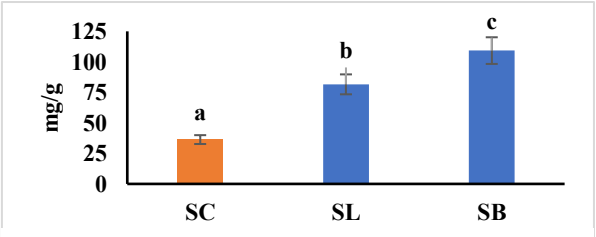

Figure 1 Concentration of total polysaccharides(mg/g) released in a wine like solution by the IDY (SC, SL, SB LSD - Least Significant Difference, Anova Fisher'test Different Letters Indicate Statistical Significances,)

been shown to have different effect on the wine colloidal stability, further researches are needed to evaluate their relevant impact.
Regarding GSH, each IDYs was able to release it in the solution in different quantities. This is an important feature, permitting to use IDYs also to possibly reduce the addition of SO_2 during the winemaking process.

3.2 Impact of IDYs in a Trebbiano white wine

All the wine samples treated with IDY showed amounts of total polysaccharides in agreement with those found in the wine like solutions. Moreover, each IDY was able to improve the protein stability of the corresponding treated wine. However, contrary to what was expected, the decrease of ΔNTU, did not not result correlated with the amount of polysaccharides. Indeed, the wine treated with IDY-SB showed the lowest values of protein stability, although it contained the highest amount of polysaccharides in the respective wine.
In comparison with the control, all treated wines presented lower absorption in the yellow wavelength, were brighter, and were less red (a* negative values *versus* the control) and less yellow (b* lower values *versus* the control), (Table 1). These results might be due to an antioxidant activity of the IDYs, or to an adsorption of the phenolic compounds by the inactivated yeasts cell. In this regard, we are conducting specific investigations in order to understand better their mechanism of reaction.

Table 1 *Absorption at 420 nm and CIELab coordinates(L*,a*,b*) of Trebbiano white wines (mean value ± standard deviation) evaluated 15 days after the addition of the inactivated dry yeast (SC, SL, SB). CT: Trebbiano wine without IDY treatment.*

Sample	A420	L*	a*	b*
CT	0.099±0.001	97.4±0.078	0.29±0.040	6.63±0.018
SC	0.084±0.005	97.9±0.362	-0.30±0.036	5.55±0.098
SL	0.084±0.002	98.0±0.100	-0.23±0.028	5.58±0.051
SB	0.076±0.001	98.7±0.044	-0.35±0.020	5.35±0.022

Regarding wine aromatic profile, each IDY have shown a different impact (Table 2); in particular, IDY-SC determined a lower decrease of higher alcohols, in comparison with both IDY-SL and IDY-SB. Interestingly, the yeast derivative IDY-SL determined no decrease of ethyl and acetates esters, compared with the control as well as with the other two yeast derivatives (IDY-SC and IDY-SB). Further investigations are currently underway to understand whether the impact on aromatic compounds is due to their interaction with the polysaccharides released in the wine or with those still present in the cell wall. On the other hand, some authors have found similar results and they hypothesized a hydrophobic interaction between specific aromatic compounds and the polysaccharides (Chalier et al, 2007).

Table 2 *Main volatile compounds of Trebbiano white wines (mean value ± standard deviation) evaluated 15 days after the addition of the inactivated dry yeast (SC, SL, SB). CT: Trebbiano wine without IDY treatment.*

Volatile compounds	CT	SC	SL	SB
Higher Alcohols (mg/L)	167.60±29.66	163.64±7.39	154.65±10.98	151.61±7.17
Carboxylic acid esters (mg/L)	50.25±7.62	36.23±4.63	44.56±1.25	38.61±6.40
Fatty acids (mg/L)	27.06±3.28	28.48±2.27	25.29±1.19	23.68±1.43
Ethyl esters (µg/L)	2861.40±544.59	2365.05±277.06	2870.31±145.75	2525.60±425.7
Acetates ester (µg/L)	3537.53±284.48	3293.87±212.16	3548.93±219.16	3122.97±504.66
Terpens (µg/L)	88.88±16.27	81.44±13.13	74.18±9.12	64.32±6.72

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Strategies to increase the sustainability of plant-based proteins

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This PhD project is focused on advancing the sustainability of agri-food chains in the production of plant-based proteins, compared to the existing products available on the market. The project aims to achieve this goal through a range of strategies, including the meticulous selection of raw materials, implementation of diverse agricultural practices, optimization of production processes, and valorization of generated by-products. The extraction of proteins will primarily focus on conventional alternative sources like Pea seeds.

Strategie per aumentare la sostenibilità delle proteine di origine vegetale

Questo progetto di dottorato si concentra sull'avanzamento della sostenibilità delle catene agroalimentari nella produzione di proteine vegetali, rispetto ai prodotti attualmente disponibili sul mercato. Il progetto mira a raggiungere questo obiettivo attraverso una serie di strategie, tra cui la meticolosa selezione delle materie prime, l'implementazione di pratiche agricole diverse, l'ottimizzazione dei processi produttivi e la valorizzazione dei sottoprodotti generati. L'estrazione delle proteine si concentrerà principalmente su fonti alternative convenzionali come i semi di pisello.

Key words: Plant based proteins, pea protein, extraction, functional properties.

1. Introduction

In accordance with the PhD thesis project previously described (ASTI,2022), this poster reports the main results of the first two activities concerning:
(A1) The characterization of different pea varieties from different production sites/years
(A2) The Application of an improved conventional extraction process (with an alkaline extraction step followed by an isoelectric precipitation step) and the investigation of the variety/site effect on extraction yield and protein functionalities.

2. Materials and Methods

2.1 Characterization of raw material

The following methods were employed to investigate various physico-chemical characteristics:

- Total dry matter content: The AOAC (1999)
- Ash content: Method 08-01 (AACC, 1984)
- Total fat content (standard Soxhlet extraction with petroleum ether after an acid hydrolysis with 4 M HCl)
- Protein content: based on the Kjeldahl method using a conversion coefficient of 6.25
- Starch content based on thepolarimetric method which determines the content of starch and high-molecular-weight starch degradation products);
- Total dietary fiber content (based on the AOAC Method 991.43 and AACC Methods 32-07-01)

2.2 Protein extraction protocol

Due to confidentiality issues with the company the PhD project is carried out in collaboration with, complete details of the protocol cannot be provided. The process involved an alkaline extraction at pH 9, followed by a centrifugation step to separate a solid residue from the liquid phase. The latter was acidified to pH 4.5 to get protein precipitation. The precipitate was then separated through a further centrifugation step.
The physico-chemical characterization was also analyzed for the extracted products (Deposit, Gel, and whey).

2.3 Statistical analysis

a statistical analysis was performed to compare means using a 1-factor analysis of variance (ANOVA) and a Tukey test. The analysis was conducted using SPSS software. The significance level was set at a threshold of $p < 0.001$.

3. Results and Discussion

The following tables report the obtained results. The values are reported as mean values ± standard deviations of the replicates (at least three). Content of ash, proteins, fat, starch and dietary fiber were reported on dry matter content. A Full characterization for the extraction products as well as the mass balance is still ongoing.

SITE/YEAR	VARIETY	TOTAL DRY MATTER (%)	ASH (%)	TOTAL PROTEIN on DM (%)	TOTAL FAT on DM (%)
FOGGIA 2021	A	90,06±0,06	3,29±0,03	22,83±1,88	2,02±0,16
	B	89,78±0,01	3,56±0,04	23,25±1,34	1,75±0,09
	C	89,90±0,03	3,20±0,16	23,01±2,27	1,5±0,11
	D	89,88±0,04	3,69±0,01	23,05±1,32	2,11±0,17
FOGGIA 2022	A	89,54±0,09	3,40±0,04	25,34±2,23	1,25±0,05
	B	89,97±0,10	3,32±0,06	23,83±1,45	1,08±0,01
	C	89,48±0,04	3,14±0,02	23,15±1,03	1,6±0,11
	D	89,63±0,03	3,42±0,02	25,45±0,97	1,58±0,02
RAVENNA 2022	A	91,57±0,24	3,52±0,06	19,05±2,03	2,78±0,15
	B	91,46±0,09	3,35±0,01	20,61±1,35	2,44±0,4
	C	91,08±0,04	3,23±0,04	19,61±2,43	1,74±0,34
	D	91,02±0,03	3,31±0,05	19,49±1,56	2±0,09
SCHIAVON 2022	A	90,38±0,09	3,22±0,07	21,82±1,84	2,75±0,16
	B	90,33±0,12	3,26±0,00	22,23±2,57	2,57±0,1
	C	90,97±0,10	3,22±0,02	20,83±0,83	2,35±0,15

SITE/YEAR	VARIETY	TOTAL STARCH (%ON DM)	TOTA DIETARY FIBER (% ON DM)
FOGGIA 2021	A	42±0,5	17,72±0,52
	B	42±0,5	18,59±0,69
	C	41±0,5	15,22±1,49
	D	40±0,5	18,25±1,10
FOGGIA 2022	A	42±0,5	17,31±1,21
	B	37±0,5	19,68±0,94
	C	39±0,5	16,75±0,48
	D	41±0,5	17,01±0,64
RAVENNA 2022	A	47±0,5	19,54±0,68
	B	42±0,5	21,43±0,21
	C	44±0,5	18,80±0,16
	D	43±0,5	19,51±0,67
SCHIAVON 2022	A	39±0,5	20,39±0,26
	B	40±0,5	20,86±0,66
	C	35±0,5	19,15±0,05

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Combining chemical and sensory data to study the acceptability of single-varietal wines (Pinot Gris and Pinot Noir) from different geographical origins

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The main objectives of the Ph.D. project are 1) The characterization of the sensory profile and the chemical profiles (volatile and phenolic compounds) of the single variety Pinot Gris and Pinot Noir wines from seven different origins. 2) The study of the chemical and sensory profiles with explorative statistical methods to see the correlation among them and to understand which were the most interesting sensory and chemical attributes influencing the overall sensory quality of the wines. The sensory test was carried out by two different regional panels.

Combinazione di dati chimici e sensoriali per lo studio dell'accettabilità di vini monovarietali (Pinot grigio e Pinot nero) di diverse provenienze geografiche

Gli obiettivi principali del progetto di dottorato sono: 1) la caratterizzazione del profilo sensoriale e dei profili chimici (composti volatili e fenolici) di vini monovarietali Pinot grigio e Pinot nero di sette diverse provenienze internazionali. 2) l'elaborazione con metodi statistici esplorativi dei dati per studiare la correlazione tra di essi e per capire quali siano gli attributi sensoriali e chimici più interessanti che influenzano la qualità sensoriale complessiva dei vini. Il test sensoriale è stato eseguito da due panel regionali diversi.

Key words: Pinot Gris, Pinot Noir, MRATA (Modified Rate-All-That-Apply), HPLC-MS, HS-SPME-GCxGC

1. Introduction

In accordance with the PhD thesis project, this poster reports the main results of the first two activities concerning:
(A1) the generated sensorial (aroma, taste and flavour) attributes of both Pinot Gris and Pinot Noir wines;
(A2) the analysed volatile and phenolic compounds and their influence in the overall sensory quality (OQJ) in both Pinot Gris and Pinot Noir wines.

2. Materials and Methods

Both Pinot Gris and Pinot Noir bottles were provided by FruitService S.r.l. (Bolzano). The sensory analysis was performed in three phases: one round table, two training sessions (1 hour each), and two MRATA (Modified Rate All That Apply) sessions (Nishida et. al., 2021). Cysensy- an SQL binding sensory analysis web software developed in collaboration with the Engineering Faculty of the Free University of Bozen-Bolzano was used during the training and the MRATA sessions. The panel was recruited on a voluntary basis and included the staff and students from the university. The panellists for Pinot Gris included 10 people, 40% male and 60% female, aged between 25 ± 2 years old; for Pinot Noir, the sensory panel included 11 people, 55% females and 45% males also aged between 25 ± 2 years old. In addition, sensory test was also performed by a local German panel in an accredited laboratory for wine testing (DIN EN ISO/IEC 17025:2018). The round table was performed using a collaborative online whiteboard (Jamboard, Google). The university panel was trained for specific aroma, taste and flavour that characterized the products in the two training sessions. For the identification of the phenolic compounds, UHPLC-DAD-QqQ/MS (Ultra High Performance Liquid Chromatography) coupled with diode array detection (DAD) and mass spectrometry detector (MS) were used according to published methods (Dupas de Matos et al., 2020). Head Space Solid-Phase Microextraction combined with Comprehensive Two-Dimensional GC coupled to Time-Of-Flight Mass Spectrometry (HS-SPME-GCxGC-ToF/MS) using a Flow-Modulated interface between the two capillary columns in a Pegasus® Flux BT 4D (LECO Corporation, Germany) was used (Poggesi et. al., 2022).

3. Results and Discussion

3.1 Sensorial attributes of Pinot Gris and Pinot Noir wines

The averaged results for the significant sensory attributes for both Pinot Gris and Pinot Noir wines are given below. For Pinot Gris the significant attributes were "green colour", "yellow colour", and "floral" aroma. Wines from New Zealand (Marlborough) had the highest score for green colour intensity. Yellow colour intensity was the

highest for wines from South Africa (irrigated land) and the floral aroma was the highest in wines from New Zealand (Gisborne).
For Pinot Noir the significant sensory attributes were "violet red colour", "red brown colour", "licorice aroma" and "warmness". Violet red colour was the most intense in the South African (Western Cape) wines and less intense in the wines from Chile (Central Valley). The "red brown colour" and "warmness" score was higher for wines from Argentina. Although the average score for "licorice aroma" was relatively low compared to other attributes, it was a significant attribute highest in the wines from New Zealand (Marlborough).

3.2 Correlation among sensory and chemical profiles

Multiple factor analysis (MFA) was used to fuse different datasets for both Pinot Gris and Pinot Noir samples that included the basic oenological parameters, sensory attributes, volatile compounds, phenolic compounds, proanthocyanidins and also anthocyanins (for Pinot Noir) into a single computation for an extrapolated overview of the correlations between the variables and the also various trends among the observations (Poggesi et. al., 2022). Only the wines and the sensory variables that correlate with the overall quality score (OQJ) are mentioned here.
For Pinot Gris, the observation plot showed well separation of wine samples from different countries along both PC1 and PC2. The wines from New Zealand (Gisborne) were characterized by attributes, such as floral aroma, stone-fruit flavour, yellow colour, caramelized aroma, and tropical aroma, which showed a positive correlation with OQJ. The German panel also preferred the wine samples from New Zealand (Gisborne), since they found them to be more characteristic of green-yellow, typical and bright, slightly fruity and aromatic and discreetly acidic attributes.
For Pinot Noir, the wine samples were not as clearly separated as Pinot Gris samples, with some wine replicates far from each other. But, the wine sample with the highest quality score according to the university panel was the wine from Chile (Central Valley) that was characterized by the sensory attributes - cloves aroma, fresh wood aroma, red fruit flavour, cherry aroma and spicy flavour. On the other hand, the German panel preferred the wines from Argentina (Mendoza) which were more characterized by attributes such as dark red, bright, subtle, and light wood aroma notes, rich, light fruit and woody flavour, and slightly tannic taste.
These preliminary observations of correlations between sensory and chemical profiles could provide useful information to both the wineries and the trader companies for making optimal decisions for marketing these products according to the acceptance behaviour of consumers around the world.

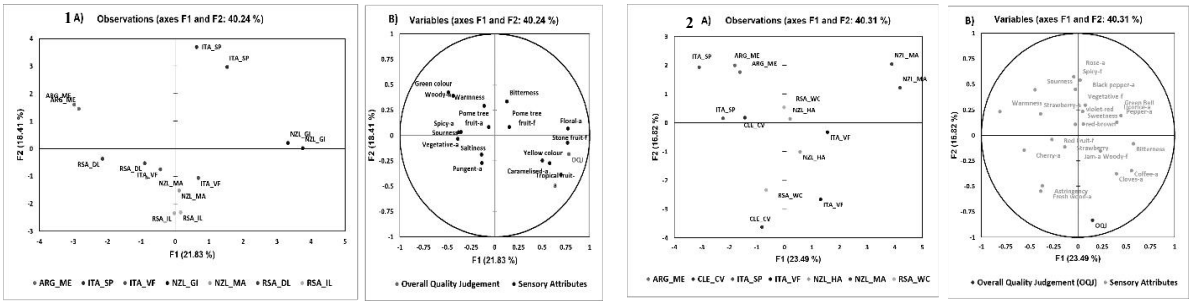

Figure 1&2 *MFA for Pinot Gris and Pinot Noir dataset. (A) shows the observation plot. (B) shows the sensory analysis variables.*

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Recyclable Packaging for Ground Coffee: Barrier Design and Permeability Studies

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During the first years of PhD a deep characterization of recyclable packaging materials for ground coffee has been faced. The first step was to study the gas (O_2 and CO_2) and water vapor (WV) barrier permeability performances of different polyolefin-based (PO-based) multilayer structures to identify their strengths and weaknesses. Different conditions of temperature (T) and relative humidity (RH) were considered for gas and vapour permeability tests under isostatic conditions, to assess the diffusion behaviour of new-generation materials. From the permeability curves it was possible to derive transport properties like diffusivity, while the temperature sensitivity of permeation was assessed by the Arrhenius model. Later, the position of the barrier layer inside the multilayer structure and its influence on permeability was investigated. A Box-Behnken Experimental Design was used to study the effect of barrier layer location, temperature, and relative humidity on O_2 permeation.

Packaging riciclabile per caffè macinato: design di barriera e studi di permeabilità

Durante i primi anni di dottorato è stata affrontata la caratterizzazione dei materiali di confezionamento riciclabili per il settore del caffè in polvere. La prima fase è stata dedicata allo studio delle prestazioni di barriera a gas e vapori di tre differenti materiali a base poliolefinica per identificarne punti di forza e di debolezza. Per le prove di permeabilità a gas e vapore in condizioni isostatiche sono state prese in considerazione diverse condizioni di temperatura e umidità relativa. Dall'elaborazione delle curve di permeabilità è stato possibile ricavare parametri utili alla comprensione del fenomeno di diffusione, mentre l'influenza della temperatura sulla velocità di permeazione è stata valutata attraverso l'applicazione del modello di Arrhenius. Successivamente, un Disegno Sperimentale Box-Behnken è stato utilizzato per studiare l'effetto della posizione dello strato di barriera, della temperatura e dell'umidità relativa sulla permeazione di O_2.

Key words: Barrier design, Polyolefin-based packaging materials, Gas Permeability, Coffee Packaging

1. Introduction

In accordance with the PhD thesis project previously described (De Agostini, 2022), this document reports some results concerning:

(A1) the characterization of new packaging materials for coffee powder in terms of barrier performances to gas and vapour. A barrier design approach has been adopted in order to build predictive models of shelf life in accordance with the protection requirements of coffee powder, as planned for later phases of the work.

2. Materials and Methods

Polyolefin-based multilayer structures with metallization (sample coded as "Met") or a polymeric thin layer as main barrier (samples 2H and 3H), were the subject of investigation. **Table 1** shows the general structure of the films and their average thickness (8% of variability).

Table 1: *Description and codification of the materials used.*

Sample Code	Multilayer structure	Thickness (µm)
Met	PP, PE, PPmet (barrier layer)	130 (± 11)
2H	PE, PE, barrier layer	128 (± 10)
3H	PE, PE barrier layer	140 (± 11)

All the materials meet legal recyclability requirements. Permeability performances to gas and moisture were investigated using the ASTM D3985 for oxygen, ASTM F2476 for carbon dioxide and ASTM F1249 for water vapour. Materials were tested through an isostatic permeability tester (Totalperm, Extrasolution, Italy) under different temperatures (25-35-45°C) and relative humidity conditions (0-65-90%). From the permeability curves it was possible to derive diffusional parameters like the half-time of permeation ($t_{1/2}$), the diffusion coefficient (D) and the acceleration factors Q_{10}. The Arrhenius model for permeability coefficient [$KP=KP_0\exp(-E_a/RT)$] was applied to estimate the activation energy for O_2 and CO_2 as seen by Schmid, 2015. The effect of barrier location (inside a PO-based multilayer structure), T and RH on O_2 permeation was studied according to a three levels Box Behnken Experimental Design. Design Expert 10 software (Stat-Ease, Inc., Minnesota, USA) was used to build

the experimental design; a response surface methodology was applied to find the optimal conditions for minimizing the gas permeability.

3. Results and Discussion

3.1 Determination of the Permeability Activation Energy

Gas Transmission Rates (OTR and CO_2TR) and Water Vapour Transmission Rate (WVTR) obtained at each condition of T and RH, were converted into the coefficient permeability KP, as described in Equation 1.

$$KP = l * Q/A * t\,(p_1 - p_2) = GTR/(p_1 - p_2) \quad (1)$$

where KP is the coefficient of permeability, l and A are respectively the thickness (µm) and the surface (m2) of the film interested in the permeation phenomena, Q is the amount of gas that passes through the packaging film (cm3), t is the time (24h), while p1-p2 is the difference of pressure (bar) between the two cells of the permeability tester (bar).

The permselectivity, i.e. the permeability ratio of KP(CO_2):KP(O_2), decreased with increasing T, at 0%RH (data not shown) because the O_2 diffusion was largely influenced by temperature. The presence of water vapor influenced the diffusivity of both CO_2 and O_2, especially for 2H and 3H materials and their permselectivity values were nearly constant with increasing T. This could be due to the hydrophilic nature of their barrier layers. The application of Arrhenius model to the coefficients of permeation calculated in different conditions of T and RH, allowed the estimation of the activation energy (E_a) for permeation (**Table 2**). For polymers in rubbery state under ambient conditions, a higher E_a indicates a higher change in permeability when temperature changes. The presence of a metallized thin layer reduced the effect of relative humidity on O_2 and CO_2, while material 3H seemed to be the structure with higher thermal sensitivity despite its higher thickness.

Table 2: *Activation Energy (Ea) for permeation at different levels of relative humidity.*

	Activation Energy (kJ mol^{-1})					
Material	Oxygen 0% RH	Oxygen 65% RH	Oxygen 90% RH	Carbon dioxide 0% RH	Carbon dioxide 65% RH	Carbon dioxide 90% RH
Met	48,6	54,4	71,7	38,2	28,9	27,0
2H	73,4	76,9	105,8	63,5	80,1	98,5
3H	83,6	94,2	119,0	57,2	89,2	108,7

The investigation about the role of barrier layer location inside the multilayer structure was tested on a polyolefin-based material, considering the rate of permeation (OTR) under different conditions of T and RH. The distance of the barrier layer (factor C) to the environment was set at 50 µm, 57,5 µm and 60 µm. Temperature (factor A) and RH (factor B) were set at three levels: 25°C, 35°C, 45°C and 0%, 45%, 90%, respectively. The combination of the variables was set following a Box-Behnken Design. The mathematical relationship of the response on these variables was approximated by the quadratic polynomial equation (Lack of Fit test not significant as the p-value was > 0,05). The factors A, C and their interactions were significant for p-value values of 0.05; instead, only the quadratic value of factor B was still significant. Decreasing the distance between the barrier layer and the environment, the OTR increased progressively under the noticeable effect of RH, reaching maximum values at 45 ºC (**Figure 1**). This means that to position a barrier layer in a multilayer with respect to the environment and, of consequence, to the food, the structure needs to be carefully designed.

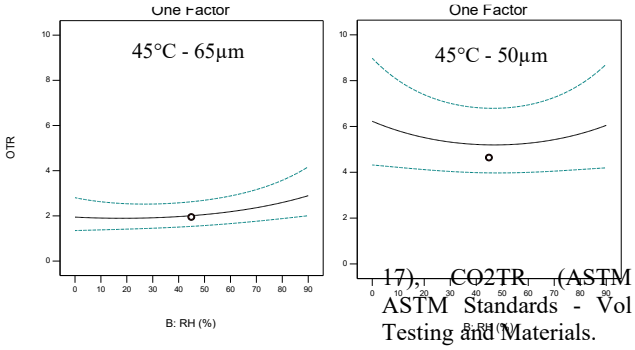

Figure 1 *Effect of barrier layer distance (65 and 50µm) on oxygen permeability (OTR) at the worst temperature tested (45°C).*

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Engineering of bioaerogels as key ingredients in the development of functional foods to deliver health through diet

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The first part of this PhD project addressed the development of aerogels from protein sources and the assessment of their suitability as functional ingredients. Both animal (whey protein isolate WPI) and vegetable proteins (soy and pea protein isolate) were considered. Protein hydrogels were prepared at different pH, ground, subjected to water-to-ethanol solvent exchange and dried by means of supercritical-CO_2 to obtain aerogels, which were characterised for physical properties. Liquid edible oils were then absorbed into aerogels, leading to oleogels. The latter were *in-vitro* digested to highlight the potentialities of aerogel-templated oleogels in modulating both protein and lipid digestibility.

Ingegnerizzazione di bioaerogel come ingredienti chiave nello sviluppo di alimenti funzionali per migliorare la salute attraverso la dieta

La prima parte di questo progetto di dottorato ha riguardato lo sviluppo di aerogel proteici e la valutazione della loro potenzialità come ingredienti funzionali. Sono state considerate proteine animali (isolato di siero di latte) e vegetali (isolati di soia e pisello). Gel preparati a diversi pH sono stati macinati, sottoposti ad una procedura di sostituzione acqua-etanolo ed essiccati mediante CO_2 supercritica. Gli aerogel ottenuti sono stati caratterizzati in termini di proprietà fisiche. Oli alimentari sono stati fatti assorbire negli aerogel, ottenendo oleogel. Questi sono stati sottoposti a digestione *in-vitro* per evidenziarne le potenzialità nel modulare la digeribilità proteica e lipidica.

Keywords: animal protein, plant proteins, porous materials, fat replacement, *in vitro* digestion.

1. Introduction

In agreement with the PhD thesis project (De Berardinis, 2022), this poster reports the main results relevant to the following activities:

(1) Identification of proteins suitable as bioaerogel precursors;
(2) Bioaerogel preparation and characterization;
(3) Application of bioaerogels as functional ingredients able to structure oil and steer lipid digestibility.

2. Materials and Methods

Whey protein isolate (WPI, Davisco Food International Inc., Le Sueur, MN, USA), soy and pea protein isolate (SPI and PPI, Myprotein, Manchester, England) were dispersed in water in concentration of 20, 14 and 19% w/w respectively, and adjusted at pH 7 or at the isoelectric pH (pI) adding NaOH or HCl (Sigma Aldrich, Milan, Italy). The dispersions were gelled at 85 °C for 10 min. The obtained hydrogels were then subjected to water-to-ethanol solvent-exchange until reaching a final ethanol concentration of 98% w/w. The obtained alcolgels were ground and supercritically dried at 12 MPa, 60 °C with a CO_2-flow rate between 80 and 120 g/min to produce aerogel particles. The latter were characterized for density, porosity, specific surface area, SEM microstructure and ability to structure sunflower oil (SO). Aerogel particles were thus used to prepare SO oleogels (Plazzotta et al., 2020), which were characterized for rheological properties. WPI aerogel particles prepared at pI and relevant oleogels were finally selected and subjected to *in vitro* digestion (INFOGEST static digestion protocol, Brodkorb et al. 2019) and visually observed *via* confocal microscopy. The digested protein fraction of WPI oleogels was characterized by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and bicinchoninic acid (BCA) assay, while lipid digested fraction by differential light scattering (DLS) and lipolysis degree.

3. Results and discussion

3.1. Identification of proteins suitable as bioaerogel precursors

The high solubility of WPI allowed to obtain 20% w/w protein solutions, which led to self-standing hydrogels upon thermal treatment at both pH 4.8 (pI) and pH 7.0. By contrast, the lower solubility of SPI and PPI accounted for a maximum dispersibility of 14 and 19% w/w, respectively. Upon thermal treatment, SPI and PPI dispersions were not able to form self-standing gels, independently of the pH. Rather, microgels in the form of spherical aggregates were obtained at their pI (4.5). Based on these results, WPI hydrogels at pI and pH 7, and SPI and PPI hydrogels at pI were selected to produce aerogels.

3.2. Bioaerogel preparation and characterization

Upon conversion into aerogel particles, highly porous powders with low density were obtained. The aerogel surface area resulted higher when proteins were conditioned far from pI (up to 350 m^2/g) (Jung et al. 2023), while much lower values were detected at pI (about 30 m^2/g). This difference can be attributed to the minimisation of electrostatic repulsions among proteins at pI, with formation of microgels with close structure. By contrast, far from pI, a stranded gel network is obtained (De Berardinis et al., 2023). As a representative example, Table 1 reports the main physical characteristics of WPI aerogel particles at pI.

Table 1. *Physical properties of whey protein isolate (WPI) aerogel at the isoelectric point. Resulting oleogel with its oil content and elastic modulus (G′) is also shown.*

WPI aerogel microstructure	Density (g/cm^3)	Surface area (m^2/g)	Oleogel appearance	Oil content (g_{oil}/100 g)	G′ ($\times 10^5$ Pa)
	0.17 ± 0.02	35.6 ± 2.3		85.2 ± 2.3	3.5 ± 0.2

3.3. Application of aerogels as functional ingredients to structure oil and steer lipid digestibility

WPI, SPI and PPI aerogel powders quickly absorbed sunflower oil, leading to highly homogeneous plastic materials, containing 85%, 72% and 63% w/w oil, respectively. As an example, Table 1 reports the appearance of the oleogel prepared with WPI aerogel particles. In all cases, oleogels showed rheological properties (G′) similar to those of commercial fats rich in saturated fatty acids. Oil structuring through aerogel particles can be explained through different mechanisms: (i) oil absorption into the aerogel pores, driven by capillary forces; (ii) oil adsorption onto the particle surface, driven by hydrophobic interactions; (iii) entrapment of liquid oil in the spaces among the aerogel protein particles, which form a network based on weak hydrophilic interactions (Selmer et al., 2019). The potentiality of aerogel-mediated oil structuring on both lipid and protein digestibility was finally studied by *in vitro* digestion. Confocal micrographs (Figure 1) clearly demonstrated that the original WPI oleogel structure was lost at the gastric level, entrapping oil droplets which were much smaller (D_{32} < 10 µm) than those observed in the case of the unstructured oil (D_{32} > 30 µm). SDS-PAGE and BCA assay confirmed that aerogelation reduced the gastric proteolysis of WP from nearly 100% to 70%. The digestion of the oleogel led to similar gastric protein digestibility. Upon intestinal digestion, aerogel proteins resulted completely hydrolysed. The lipolysis degree of the oleogel (75%) was higher than that of the unstructured sunflower oil (66%), due to the larger surface offered by smaller oil droplets to the action of intestinal lipases. This was confirmed by dynamic light scattering, showing a shift towards smaller size in the digestive micelle distribution of oleogels at the end of the intestinal phase (Plazzotta et al., 2022).

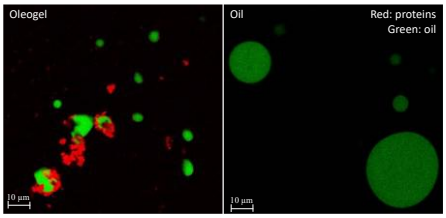

Figure 1. *Confocal microscopy after gastric digestion of sunflower oil and oleogel prepared with whey protein aerogel particles at pI.*

3.4. Conclusions

Whey, soy and pea protein isolates were demonstrated to be suitable precursors to produce aerogel powders with high porosity and surface area. The developed aerogel powders showed high oil structuring ability, leading to semi-solid materials resembling traditional fats. Oleogelation through aerogel-template approach was shown to steer both protein and lipid digestibility. These results support the possible role of aerogels as key ingredients in the development of foods able to deliver health through diet.

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Enzyme Biotechnology to Recovery Byproducts from Agricultural and Food Wastes

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This PhD project aims to find solutions to reduce wastes from agri-food processes by using enzymes as "green" tools in order to obtain added value compounds to put in new formulations. Among these wastes, hemp shives, roots from aquaculture and fish waste were individuated. Related both to the economic interest of some companies and on the availability of the product hemp shives have been selected. Due to the lignocellulosic nature of the waste, carbo-hydrolytic enzymes should be the best candidates to disassemble the matrices. Until now xylanase has been tested obtaining interesting results.

Biotecnologie enzimatiche per il recupero di byproducts da scarti agroalimentari

Questo progetto di dottorato mira a trovare soluzioni per ridurre gli scarti dei processi di produzione e trasformazione agroalimentare, utilizzando gli enzimi come strumenti "green" al fine di ottenere composti a valore aggiunto da inserire in nuove formulazioni. Tra questi scarti sono stati individuati canapuli di canapa, radici e scarti di pesce. In relazione sia all'interesse economico di alcune aziende che alla disponibilità del prodotto, sono stati scelti i canapuli. A causa della natura lignocellulosica del materiale, gli enzimi carbo-idrolitici dovrebbero essere i migliori candidati per degradare tali matrici. Finora è stata testata la xilanasi che ha prodotto risultati interessanti.

Keywords: circular economy, enzymes, hydrolases, agri-food waste

1. Introduction

In accordance with the PhD thesis project, this poster reports the main results of the following activities:
(A1) characterization of agri-food waste.
Among residues and wastes, the interest of the project was focused on fish waste, roots from aquaculture facilities and hemp shives. Each one was subjected to a proximal characterization and, after an evaluation in terms of economic and ecological sustainability, hemp shives were chosen for the following experiments. Lignocellulosic materials are made of cellulose, hemicellulose and lignin, in different proportions regarding to the vegetal species (Lin *et al*., 2010). Thanks to enzymatic biotechnologies it is possible to recover cellulose and other compounds in order to give them a new commercial life and reduce the amount of deforestation related with cellulose requirements by industry.
(A2) Choice of the best enzymatic strategy
To extract cellulose following enzymatic approach it is required the application of an hemicellulase which could be pectinase, xylanase (Covino *et al*., 2020) or a mix of both of them. Generally, for wood substrates, it is better to use xylanase (Alvarez *et al*., 2016). Until now, Xylanase from *Bacillus subtilis* has been tested in different essay conditions. Furthermore, several washing pretreatments have been performed to make substrate more accessible for the enzyme: particularly two organic solvents (acetone and ethanol) as well as cold (25°C) and boiling (100°C) water have been tested.

2. Materials and Methods

Hemp shives were provided by local farm, while fish waste and roots from aquaculture facilities came from Department of Agricultural Sciences, University of Naples, Federico II.
Proximate characterization was made as follow: hashes with a furnace set at 550°C for 5.5 hours, Soxhlet warm extraction and Kjeldahl's method have been applied to evaluate extractable lipids and protein content respectively. The overall composition of hemp shives was evaluated by a chemical extraction (Ayeni *et al.*, 2015). Hemp shives material was subjected to different whashing pre-treatments: acetone and ethanol for 4 h, cold (25°C) and boiling (100°C) water for 30 min, then samples have been centrifuged at 10000×*g* for 10 minutes. The pellet was enzymatically treated with xylanase from *Bacillus subtilis* (Creative Enzymes, Shirley, NY, USA) in phosphate buffer (pH 6) at 65°C for 18 hours, at a constant stirring of 150 rpm in an Orbital Shaker (Forma Scientific 420). After the incubation, samples were centrifuged again in the same conditions and the pellet was dried in an oven at 105°C until constant weight; Dubois' analysis was performed on the supernatant.

3. Results and Discussion

3.1 Characterization of agri-food waste

The proximate composition of the different waste matrices is reported in Table 1. As expected, fish showed a higher amount of proteins in comparison with vegetal matrices, where polysaccharides were most abundant.

Table 1 *Proximate composition of considered food wastes.*

Waste	*Protein*	*Carbohydrate**	*Fat*	*Ash*
fish	8.7±1.6	n.d.	24.2±7.2	14.1±2.1
roots	3.9±1.4	72.4±0.4	0.6±0.1	23.1±0.7
hemp shives	4.5±1.4	89.9±0.2	0.5±0.2	5.1±0.8

*Calculated as complement to 100%; n.d., not determined.

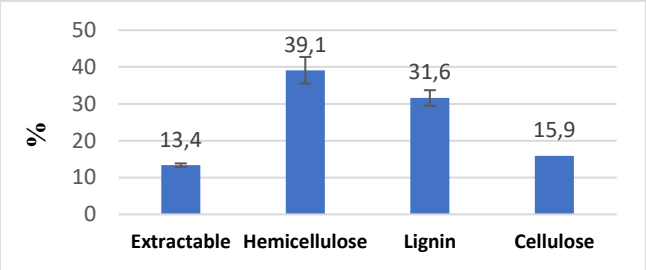

Figure 1 *Hemp shives components by chemical fractionation.*

Since pulp and paper industries have a big impact on the environment, after first characterization, most of the activities were focused on hemp to obtain cellulose from, in order to positively affect deforestation. The overall composition of hemp shives is reported in Figure 1 and results showed a remarkable amount of hemicellulose and lignin, but still an interesting percentage of cellulose (about 16%).

3.2 Enzymatic treatment

The enzymatic action of xylanase was tested on hemp material as such or subjected to pre-treatment with acetone, ethanol and water, both at room temperature and boiling. After incubation, the solid material was collected by centrifugation and characterized for organic and ash content. The residual organic material was taken as indicator of enzyme activity, assuming that the higher the hydrolytic activity, the lower the residual organic matter should be. The results showed that the efficacy of the enzyme was significantly increased when the material was subjected to boiling (30 min at 100°C) (Figure 2). Probably, the action of heat allows a better opening of the polymeric matrix which facilitates accessibility for the enzyme to the substrate.

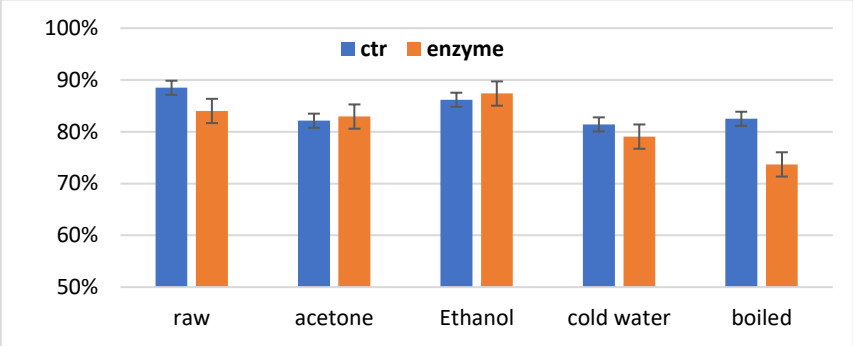

Figure 2 *Residual organic material from hemp shives raw or pre-treated as indicated in the figure, after enzymatic incubation with xylanase.*

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The effect on phenolic, aromatic and sensorial composition of wine of a pre-fermentative technique: cold liquid stabulation

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Composition and final quality of wines depend on several factors, particularly to the ability to fully exploit the qualitative potential of grape. Innovative oenological practices and different winemaking techniques are frequently used, during the wine production processes, to modify the phenolic profile and to increase the extraction of volatile components (VOCs).

L'effetto sulla composizione fenolica, aromatica e sensoriale del vino di una tecnica pre-fermentativa: La stabulazione liquida a freddo

La composizione e la qualità finale di un vino dipendono da diversi fattori. In particolare dalla capacità di sfruttare al meglio il potenziale qualitativo dell'uva a disposizione. Pratiche enologiche innovative e l'utilizzo di diverse tecniche di vinificazione durante il processo produttivo sono spesso utilizzate per migliorare il profilo fenolico e per aumentare l'estrazione dei composti volatili (VOCs).

Key words: pre-fermentative technique, phenolic compounds, volatiles compounds, antioxidant power

1. Introduction

In accordance with the PhD project (De Paolis, 2022) previously described, this study shows the preliminary results of the activities concerning the application of the cold liquid stabulation (CLS), an innovative productive process in white winemaking, applied on Arneis and Cortese grape. CLS plans to leave musts, after pressing, on their lees, kept suspended, at a low temperature (0-8°C) for a variable time (Philipp *et al*., 2022; Seabroock *et al*., 2018). The aim is the improvement of the phenolic, colour, and antioxidant features of the produced wines, as well as increasing the extraction of volatile compounds from grape skins and pulp during the process.

2. Materials and Methods

Two white musts, from Italian grape varieties 'Arneis' and 'Cortese', after destemming, crushing and pressing, have been maintained on their lees, manually suspended twice a day, at low temperature (4°C). Three periods of stabulation (three replicates each) have been tested: 7, 14 and 21 days and compared to a control without stabulation. At the end of treatment period, musts have been raked and inoculated for the alcoholic fermentation (AF). The evolution of the parameters is been analysed in three different moments (*i.e.* at the end of stabulation, at the end of AF and one month after bottling). The following basic parameters have been investigated: Brix (Brix refractometer Atago palette 0-32 Brix), pH (Inolab 730 calibrated pHmeter, WTW, Weilheim, Germany), total acidity (OIV-MA-AS313-01 method), organic acids, ethanol and glycerol by HPLC (Agilent Technologies, Santa Clara, USA), following the method proposed by Giordano *et al.*, (2009). Total phenolic index (TPI) was evaluated by measuring absorbance at 280 nm of the sample diluted in water as well as the antioxidant capacity (DPPH) (Romanet *et al.* in 2019). CIEL*a*b* parameters were evaluated according to the OIV-MA-AS2-11. Free and glycosylated volatile compounds have been studied through GC-MS analysis (Giacosa *et al*., 2019). Sensory analysis on the wines produced was carried out with a trained panel by a mixed approach of descriptive analysis (DA) and Check-all-that-apply (CATA) strategies.

3. Results and Discussion

3.1 Impact of treatment on chemical-physical parameters

The CLS has an impact for both varieties on the acidic composition. The low temperature, already after 7 days, affected the content of tartaric acid, lowering significantly the total acidity at the end of stabulation for the Arneis musts and at the end of AF for Cortese. Also, pH had a significative decrease after the treatment, reaching the

Table 1 *Principal base parameters of Arneis and Cortese after treatment*

	ARNEIS After settling/stabulation					CORTESE After settling/stabulation				
	Control	P07	P14	P21	*Sign.*	Control	P07	P14	P21	*Sign.*
Total acidity (g/L of tartaric acid)	3.6±0.1 a	3.4±0.0 b	3.4±0.0 b	3.3±0.0 b	**	3.6±0.0 a	3.1±0.0 a	3.2±0.7 a	3.4±0.0 a	*N.S.*
pH	3.18±0.01 ab	3.19±0.00 a	3.18±0.00 b	3.16±0.0 c	***	3.19±0.00 a	3.17±0.00 b	3.16±0.00 c	3.16±0.00 c	***
Tartaric acid (g/L)	4.40±0.03 a	4.08±0.02 b	3.98±0.04 c	3.98±0.02 c	***	4.55±0.03 a	3.72±0.02 c	3.83±0.04 b	3.58±0.00 d	***

lowest values after 21 days of stabulation (Tab 1). In the wines analysed after bottling, TPI remained lower in

Cortese stabulated samples meanwhile in Arneis the trend changed with higher TPI in the treated samples. DPPH had the same behaviour in Arneis, while in Cortese it was not affected by the stabulation (Tab.2)

Table 2 *TPI and DPPH values of Arneis and Cortese wines one month after bottling*

	ARNEIS Bottled wine					CORTESE Bottled wine				
	Control	P07	P14	P21	*Sign.*	Control	P07	P14	P21	*Sign.*
TPI (mg gallic acid/L)	6.2±0.0 c	6.1±0.0 c	6.4±0.0 b	6.6±0.1 a	***	6.3±0.1 a	6.2±0.1 a	5.9±0.0 b	5.8±0.0 b	***
DPPH (mmol Trolox/L)	1.30±0.01 bc	1.28±0.01 c	1.34±0.01 ab	1.38±0.03 a	**	1.99±0.02 a	1.95±0.01 a	1.96±0.02 a	1.91±0.05 a	*N.S.*

3.2 Impact of treatment on sensory analysis and volatile compounds

Both the Arneis and Cortese wines did not show differences in terms of bitterness, astringency, acidity and body due to the CLS. For Arneis, the control wine had *grapefruit*, *rose* and *lime* as the highest cited descriptors, after 7 days tropical fruits-related descriptors, while after 14 days *apple*, *pear* and *honey* were the most cited. At P21 the more intense descriptors were *lemon* together with floral hints. For Cortese, *green apple* and *pineapple* have been used to describe control and 21-days sample. The descriptors *pear* and *rose* were characterising the P14 and P21 wines, whereas the P07 was different, characterized by *honey* and *peach* notes. Nevertheless, in Cortese an increasing trend for liking for the stabulated wines was found, with P14 and P21 preferred than control. Regarding the volatile composition of wines (Tab. 3), in Arneis control and P07 had higher content of esters and volatile acids. Same for norisoprenoids and volatile phenols, in particular the latter decreased with the increasing of stabulation length. Instead terpenes, higher alcohols, volatile sulphur compounds and benzenoids are higher in the most stabulated. In Cortese wines less variations among samples has been found. Indeed, no significative differences were in esters, higher alcohols, terpenes, volatile acids, norisoprenoids and benzenoids. There are significative decreases, in most stabulated wines, for C6-compounds and volatile sulphur compounds. Meanwhile a higher value of volatile phenols has been registered for the 21-days stabulated sample. These results highlighted a different impact of CLS treatment depending on the variety.

Table 3 *Aromatic composition of Arneis and Cortese wines one month after bottling*

ARNEIS					
	Control	P07	P14	P21	*Sign.*
Acetate esters	5716.22±60.39 a	5832.82±75.05 a	4792.31±23.78 b	4619.71±126.20 b	***
Ethyl esters	16153.1±670.70 a	1631.2±106.26 a	14917.29±367.57 b	14613.67±453.66 b	*
Higher alcohols	11848.03±163.37 c	12255.85±68.28 bc	12857.01±214.25 a	12712.76±287.38 ab	**
Volatile acids	10413.86±201.54 a	10617.14±182.58 a	9577.6±37.33 b	9770.83±155.72 b	**
Volaitle sulfur compounds	224.56±9.76 b	209.44±4.26 b	226.68±6.28 b	264.88±19.01 a	*
Terpenes	4.1±0.81 c	4.79±0.00 bc	5.8±0.52 ab	6.84±0.37 a	**
Norisoprenoids	14.64±1.36 a	12.86±0.67 a	9.83±0.45 b	10.1±0.17 b	**
Volatile phenols	1038.69±54.95 a	878.2±40.79 b	743.58±10.14 c	786.7±39.53 bc	***
Benzenoids	12221.26±531.98 c	12248.28±45.41 bc	13217.35±20.73 b	14360.34±457.65 a	**
CORTESE					
	Control	P07	P14	P21	*Sign.*
Acetate esters	4924.28±291.65 a	4036.23±475.69 b	5429.23±139.58 a	5352.82±138.33 a	**
C6 compounds	1544.77±35.52 a	1408.49±108.86 ab	1366.29±27.62 b	1363.08±15.58 b	*
Volaitle sulfur compounds	191.12±2.08 a	144.39±17.74 b	145.87±10.98 b	146.73±2.81 b	**
Volatile phenols	1057.46±7.56 b	1107.47±127.11 ab	1157.7±33.37 ab	1259.73±61.74 a	*

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Health-promoting protein-derived peptides in functional foodstuff

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The present PhD thesis's main objective is to develop tailored protein hydrolysates from food industry by-products to be used as innovative functional food ingredients. Milk whey and pea proteins were used as animal and vegetal protein sources respectively. Two of the activities included in the PhD thesis project are described. Firstly, the protein hydrolysates were characterized in terms of the degree of hydrolysis and molecular weight distribution. Secondly, the protein hydrolysates' technological and *in vitro* biological properties were assessed.

Impiego di peptidi bioattivi ottenuti dall'idrolisi delle proteine in alimenti funzionali

Il principale obiettivo di questa tesi di dottorato è lo sviluppo di idrolizzati proteici con specifici attributi, ottenuti da sottoprodotti delle industrie alimentari, che possano essere utilizzati come ingredienti funzionali. Le proteine del siero di latte e le proteine di pisello sono state usate come proteine di origine animale e vegetale, rispettivamente. Vengono descritte due delle attività del progetto di tesi di dottorato. Inizialmente gli idrolizzati proteici sono stati caratterizzati in termini di grado di idrolisi e distribuzione dei pesi molecolari. Inoltre, sono state valutate le proprietà tecnologiche e biologiche degli stessi.

Key words: whey proteins, protein hydrolysates, technological properties, biological properties

1. Introduction

In accordance with the PhD thesis project previously described (Di Filippo, 2022), this poster reports the main results of the two activities concerning:

(A1) the **physiochemical characterization of whey protein hydrolysates** in terms of the degree of hydrolysis (DH), and molecular weight distribution;

(A2) the **assessment of whey protein hydrolysates' technological properties**, namely solubility, emulsifying, foaming and gelling properties, and *in vitro* **biological** property (antioxidant capacity).

2. Materials and Methods

Whey protein hydrolysates (WPHs) were obtained from a whey protein isolate (WPI) dissolved in potassium-phosphate through a hydrolysis process conducted with the use of Alcalase 2.4L or Protamex enzymes for different times, from 10 to 480 minutes, at 50 and 55°C respectively. WPHs were freeze-dried and further characterized in terms of the degree of hydrolysis, with the o-phthaldialdheyde (OPA) assay (Donkor et al., 2007), whereas the percentage molecular mass (MW) distribution was determined through HPLC analysis using a TSKgel column (Cui et al., 2022). Peptides were classified according to their MW into three groups (I group>18 kDa, II group18-3 kDa, III group 3-0.6 kDa, and IV group <0.6 kDa). Technological properties were then assessed. Solubility was performed by drying the insoluble precipitate of WPI and WPHs, solubilized in water, in a vacuum oven overnight. The foaming ability and stability indices were determined by placing 10 mL of WPI and WPHs samples, overnight solubilized in distilled water (1%), in a graduated cylinder, generating foam with an Ultraturrax® at 800 x g for 3 min and measuring it over 1 h. Similarly, WPI and WPH solutions were assayed for emulsion ability and stability with the addition of sunflower oil. After homogenization with Ultraturrax® at 1000 x g the emulsions were spectrophotometrically analysed, and the indices were calculated by the equations reported by Alonso-Miravalles et al. (2019). The gelling property was determined according to Zhao et al. (2017). WPI and WPHs were solubilized in distilled water at 5, 10, and 20% protein concentration, heated at 90 °C for 30 min, cooled at 4 °C and tested for viscoelastic properties using a rheometer (ThermoScientific RheoStress, Haake, Germany). Regarding biological properties, WPHs were tested for DPPH scavenging activity. Statistical analysis was performed by analysis of variance (one-way ANOVA) and Tukey's comparison test.

3. Results

WPHs showed an increase in the degree of hydrolysis (DH) as the time of hydrolysis increased (Figure 1a). After 480 min, WPHs obtained with Alcalase and Protamex enzymes reached a comparable DH (25.65% and 26.71%, respectively). The DH increasing trend, however, depicted a statistical difference after the first 10 min of hydrolysis since Alcalase demonstrated a faster proteolytic activity as compared to Protamex. This observation was also confirmed by the hydrolysates' percentage molecular weight (MW) distribution (Figures 1b and 1c).

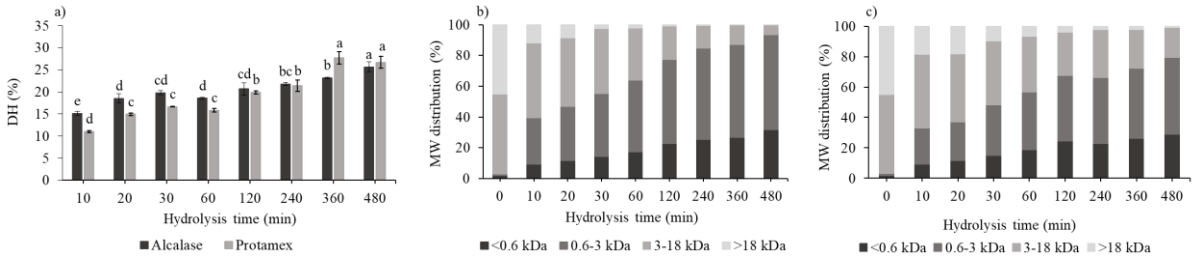

Figure 1 *Degree of hydrolysis (DH) (a) and molecular mass (MW) distribution of Alcalase (b) and Protamex (c) WPHs. [a-e]: for each enzyme, means indicated by different letters are statistically different ($p<0.05$).*

Technological properties were then evaluated. Table 1 reports the solubility, foaming and emulsifying ability indices (FAI and EAI) of WPHs. The increase in hydrolysis time revealed an increase in solubility just after 10 min, when hydrolysis was conducted with both enzymes, as compared to WPI. A difference was observed in Alcalase-WPHs that, after 60 min hydrolysis, demonstrated a slight solubility reduction whereas the solubility of Protamex-WPHs maintained constant values throughout the entire hydrolysis time. An increased solubility is the result of a favoured exposure of hydrophilic groups on the surface that could enhance WPHs-water interaction. Foaming properties were thus analysed and as expected, FAI of WPHs was higher compared to WPI. Again, hydrolysis conducted with Alcalase promoted an increase in FAI from 10 to 30 min hydrolysis, whereas Protamex-WPHs exhibited a progressive FAI increase upon hydrolysis. Foaming stability (FSI) was detected to gradually increase, regardless of the enzyme used. Both solubility and foaming ability results could be mostly attributed to the variation in MW distribution (Figures 1b and 1c). The initial production of fractions with lower MW than native proteins during the first minutes of hydrolysis could have led to peptides being more prone to displace themselves at the water-air interface. However, this behaviour caused a decrease in the EAI and the emulsifying stability index (ESI) of WPHs, compared to WPI, due to the lower ability of short chains in reducing the interfacial tension. Alcalase WPHs were not able to form a gel network, while Protamex WPHs formed a gel at 10-, 20- and 30-min hydrolysis. Such results could be again attributed to the different trends in peptide dimensions obtained by the two enzymes. Finally, the *in vitro* antioxidant activity displayed a similar increasing trend until 120 min for Alcalase (88.11% ± 0.77) and 60 min Protamex hydrolysis (80.65% ± 0.33). By further increasing hydrolysis time, Protamex-obtained WPH's antioxidant activity remained unchanged, whereas Alcalase-WPHs showed a decrease in antioxidant activity to 48.85% ± 1.92. However, WPHs were demonstrated to enhance the antioxidant activity of WPI (26.33% ± 2.18). Such results highlight the possibility to obtain, through the selection of appropriate enzyme and hydrolysis degree, the best tailored WPH-based ingredients in terms of technological and/or biological properties.

Table 1 *Solubility, foaming ability index (FAI) and emulsifying ability index (EAI) of WPHs.*

Hydrolysis time (min)	Alcalase			Protamex		
	Solubility (%)	FAI (%)	EAI (m^2/g)	Solubility (%)	FAI (%)	EAI (m^2/g)
0	88.18±0.80^{d}	20.4±3.9^{f}	2.27±0.44^{a}	88.18±0.80^{c}	20.4±3.9^{d}	2.27±0.44^{a}
10	92.26±0.70^{ab}	76.2±3.0^{e}	1.19±0.12^{bce}	92.20±0.91^{b}	72.2±2.6^{c}	1.82±0.43^{ab}
20	91.81±0.64^{ab}	129.6±0.0^{b}	1.34±0.25^{bcd}	92.59±0.87^{a}	100.3±10.9^{bc}	1.66±0.40^{ab}
30	93.36±0.85^{a}	176.5±4.9^{a}	1.60±0.16^{ab}	91.59±0.81^{ab}	104.0±5.7^{b}	1.05±0.22^{b}
60	92.48±0.52^{c}	139.7±3.7^{b}	1.50±0.09^{ac}	92.39±1.04^{a}	104.0±5.7^{b}	1.19±0.30^{ab}
120	90.25±0.72^{bc}	94.4±2.5^{d}	1.49±0.02^{bc}	91.83±0.95^{ab}	136.0±5.7^{a}	1.11±0.05^{ab}
240	90.50±0.11^{d}	111.7±4.2^{c}	0.80±0.03^{ce}	92.11±0.62^{a}	120.0±10.0^{ab}	0.97±0.02^{b}
360	90.32±0.48^{c}	106.0±2.8^{cd}	0.50±0.03^{e}	92.18±0.12^{a}	108.0±17.0^{ab}	1.03±0.38^{b}
480	90.25±0.65^{c}	102.2±3.1^{cd}	0.57±0.20^{de}	90.95±0.58^{ab}	122.0±19.8^{ab}	1.30±0.10^{ab}

Data show mean values (±SD, n=3). WPI data were reported for both enzymes to allow the comparison with WPHs.
[ae]: in the same column, means indicated by different letters are statistically different ($p<0.05$).

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Sustainable Wine Production Using Disease Resistant Hybrid Grape Cultivars in South Tyrol

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The first activities of this PhD are described. The primary project examines the advantages and disadvantages for winemaking of disease resistant hybrid grape cultivars (DRHGCs), also known as 'PIWI' grapes (German, *Pilzwiderstandsfähige Rebsorten*). A literature review on this topic was published in the first year. Sensory and chemical analyses were conducted in the second year. Wines produced from DRHGCs in South Tyrol were found to be distinctive in terms of sensory and chemical characters, with the anthocyanin profile of wines produced from DRHGCs being particularly distinctive.

Produzione sostenibile di vino con vitigni ibridi resistenti alle malattie in Alto Adige

Vengono descritte le prime attività di questo progetto di dottorato. Sono stati esaminati i vantaggi e gli svantaggi, per la vinificazione, delle uve provenienti da vitigni ibridi resistenti alle malattie, note anche come uve 'PIWI' (dal tedesco *Pilzwiderstandsfähige Rebsorten*). Nel primo anno è stata pubblicata una review sull'argomento. Le analisi sensoriali e chimiche condotte hanno mostrato che i vini PIWI ottenuti in Alto Adige si distinguono in termini di caratteri sensoriali e chimici, con un profilo antocianico particolarmente caratteristico.

Key words: PIWI wines; sensory profile; chemical profile; disease resistant grape varieties.

1. Introduction

This poster reports the main results of the first activity of the PhD project:

(A1) the literature concerning the chemistry of disease resistant hybrid grape cultivars (DRHGC) and the optimisation of winemaking methods for DRHGCs;

(A2) the unique chemistry and sensory profiles of wines produced using DRHGCs in South Tyrol, and how these properties influence consumer preferences.

2. Materials and Methods

Wines produced in South Tyrol using both conventional *Vitis vinifera* cultivars and DRHGCs were purchased from or donated by local producers. DRHGC wines were contrasted with conventional wines from the same producer, to ensure similar winemaking methods. Wines were assessed by a semi-trained panel using both the rate-all-that-applies (RATA) method and the projective mapping (napping) method. Wines were analysed using LC-MS (Darnal et al., 2023a, 2023b; Poggesi et al., 2022), GC×GC-MS (Poggesi et al., 2022), and a multiparametric analyser. Statistical analyses were carried out using XLSTAT and GNU R. Both Principal Component Analysis (PCA) and Analysis of Variance (ANOVA) were used.

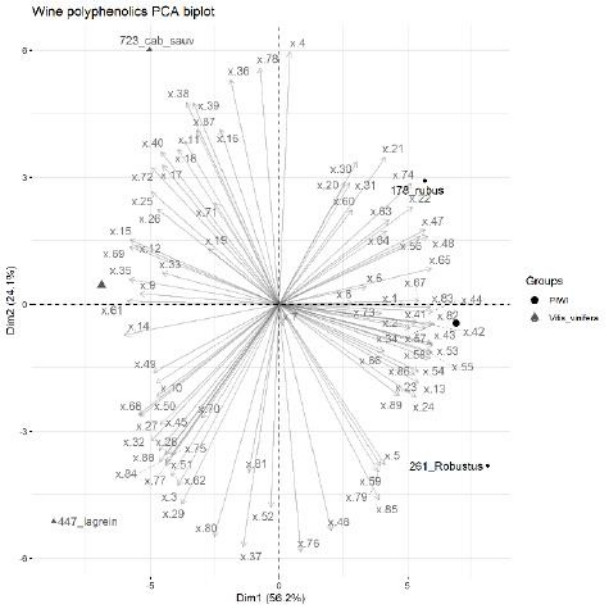

Figure 1 *PCA of the polyphenolic profile of two conventional wines ('Cabernet Sauvignon' and 'Lagrein') and two DRHGC wines (both blends of 'Monarch' and 'Cabernet Cortis'). Numbered variables (starting with 'x') represent non-volatile phenolic compounds; later work will provide precise identifications.*

3. Results and Discussion

3.1 Preliminary analytical data

The differences in chemical profile between DRHGC wines and conventional wines was discussed in detail in our literature review (Duley et al., 2023).

The anthocyanin profile of DRHGC wines is particularly distinctive due to the presence of diglucosidic and triglucosidic anthocyanins that are rare

(Boselli et al., 2008) or not present in *Vitis vinifera* (Mazzuca et al., 2005; Wojdyło et al., 2018). Future work will include sampling additional wines to achieve a more complete overview of the anthocyanin profile in PIWI wines. The profile of other phenolic compounds in DRHGC red wines was also distinctive, with PCA able to separate DRHGC and conventional wines (**Figure 1**). This is in agreement with the literature (Duley et al., 2023; Springer & Sacks, 2014). Future work with a larger sample size is necessary for further confirmation.

3.2 Sensory analysis: preliminary

The first sensory panel compared two conventional wines ('Kerner' and 'Pinot gris') and one DRHGC wine (80% 'Bronner' & 'Souvignier gris' with 20% 'Sauvignon blanc'), and was able to distinguish between DRHGC and conventional wines (**Errore. L'origine riferimento non è stata trovata.**). In particular, the DRHGC wine was associated with green colour, olfactory green bell pepper and earthy, and gustatory green bell pepper and astringency.

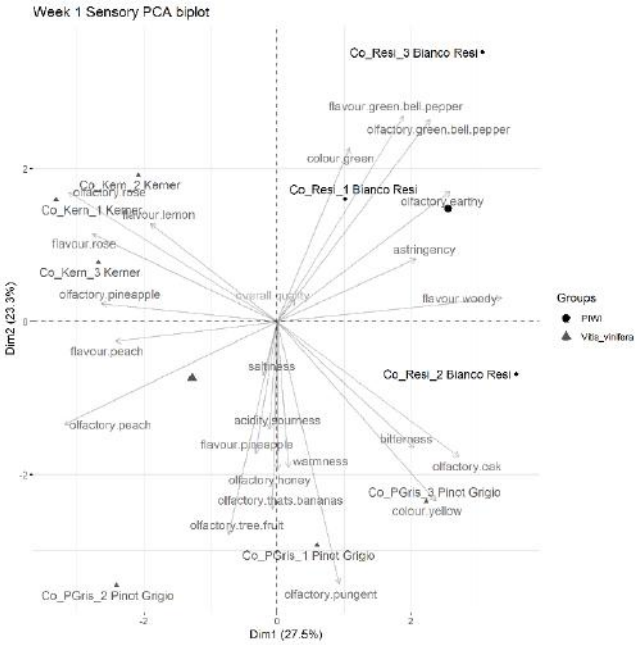

***Figure 2**. PCA of sensory data from first sensory panel. The two conventional wines tested were 'Kerner' (Co_Kern) and 'Pinot grigio' (Co_PGris), and the DRHGC wine tested was a blend (Co_Bianco_Resi, blend described above).*

For this initial sensory analysis, the preference other studies have seen for *Vitis vinifera* cultivars (Fuentes Espinoza et al., 2018; Nesselhauf et al., 2019) was not observed, in fact there was no significant difference (p = 0.264, ANOVA) in the overall quality rating between DRHGC and conventional wines observed in our study. This may indicate that DRHGC wines are becoming more accepted by wine drinkers, but will need to be confirmed with future sensory analysis sessions.

Future work will examine further wineries and cultivars and will include further white and red wines of both types to improve the awareness regarding the quality potential of these sustainably produced wines.

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Table Errore. Solo documento principale. Nutritional table of soybean strips.

Authentication of Hay Milk and its Dairy Products with Nuclear Magnetic Resonance Spectroscopy

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Co-tutors: Dr. Michael Oberhuber, Dr. Peter Robatscher, Dr. Ksenia Morozova

The EU product specification of Haymilk does not include an official methodology on its authenticity validation. Accordingly, this work proposed ^{1}H-NMR for determination of cyclopropane fatty acids (CPFAs) in milk as a molecular marker for Haymilk authenticity. The presence of CPFAs was detected in milk samples from maize or grass silage feeding with 97% and 76% accuracy, respectively, whilst authenticity of Haymilk samples were confirmed with 100% accuracy. The results of this approach were validated by GC-MS analysis. NMR revealed advantageous compared to GC-MS due to accurate determination of CPFAs with unique signal in the up-field region in NMR spectra.

Autenticità di Latte Fieno e suoi derivati con spettroscopia di risonanza magnetica nucleare ad alta risoluzione

La specificazione "latte fieno" non include una metodologia ufficiale per la validazione della sua autenticità. Questo lavoro propone ^{1}H-NMR per la determinazione di acidi grassi ciclopropanici (CPFAs) nel latte come marker molecolare per l'autenticità del latte fieno. La presenza di CPFAs è stata confermata nei campioni di latte da alimentazione insilata a base di mais o erba rispettivamente per 97% e 76%, mentre l'assenza nei campioni di latte fieno per 100%. Tutti i risultati sono stati validati tramite GC-MS. Rispetto GC-MS, NMR si è rivelata più vantaggiosa per la determinazione accurata di CPFA dovuta allo specifico segnale nello spettro NMR.

Key words: cyclopropane fatty acids, nuclear magnetic resonance, food authenticity.

1. Introduction

In accordance with the PhD thesis project previously described (Eltemur, 2022), this poster reports the main results of the first two activities concerning:
(A1) development of a 1H-NMR methodology for the determination of cyclopropane fatty acids (CPFAs) in milk as a molecular marker for Haymilk authenticity;
(A2) development of an untargeted ^{1}H-NMR fingerprinting approach to validate the authenticity of Haymilk.

2. Materials and Methods

Lyophilization of 245 milk samples was completed using a pilot plant (Martin Christ, Epsilon 2-6D LSC plus freeze-dryer, Osterode, Germany). 1H-NMR analysis of freeze-dried samples was performed both in CDCl3 and D2O solvents. All NMR experiments were carried out using a 600 MHz spectrometer (JNM-ECZ from JEOL Ltd., Tokyo, Japan), equipped with a "Royal" HFX/FGSQ probe. NMR spectra were acquired at room temperature 298 K (25 °C) with 32.000 complex points, using a 45° pulse length and 20 s of relaxation delay. A total of 1024 scans were acquired with a spectral width of 15 ppm and an acquisition time of 4 s. The spectra evaluation and processing have been carried out using Delta NMR processing and control software (v.5.3.1. Jeol Resonance Inc.). GC-MS analysis was performed on the fat extract of the milk samples after transesterification as a validation method using Shimadzu QP2010 SE GC-MS (Shimadzu, Kyoto, Japan).

3. Results and Discussion

3.1 Determination of CPFAs in milk as a molecular marker using targeted 1H-NMR

The CPFAs show a characteristic quartet signal in the chemical shift region between -0.30 and -0.36 ppm corresponding to the cis-methylene proton in the cyclopropane ring (Figure 1) (Knothe, 2006). Such upfield region does not overlap with any other signal arising from milk sample, therefore, it enables a precise detection and thus quantification of the CPFAs (Lolli *et al.*, 2018).

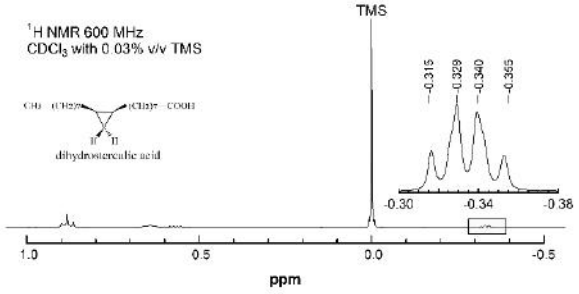

Figure 1 *600 MHz [1]H-NMR spectrum of CPFA standard (dihydrosterculic acid) in $CDCl_3$. The signal of TMS internal standard was fixed to 0 ppm. The signal resonates from CPFA was expanded for identification.*

The CPFA signal at -0.34 ppm was selected for quantification using the signal of TMS as reference. The concentration of CPFAs in freeze-dried milk sample (expressed mg of CPFA/kg of milk fat) was calculated as described by Eq.1:

$$(CPFA) = [TMS] \times \frac{A_{CPFA}}{A_{TMS}} \times 12 \times \frac{MW_{CPFA}}{m_{milk}} \times F \quad (1)$$

Where, [TMS] is the concentration of the internal standard (2.20 mM), A_{CPFA} and A_{TMS} correspond to the areas of CPFA and TMS signals, respectively, MW_{CPFA} is the molar mass of dihydrosterculic acid (294.489 $g \cdot mol^{-1}$), m_{milk} is the mass of freeze-dried milk sample (50 mg), and finally F is factor converting the concentration of CPFAs from mg/g freeze dried milk to mg/kg of milk fat. According to the calibration curve of CPFA standard solution (matrix effect was negligible), the area of the quartet at −0.34 ppm was linearly related to the concentration of CPFAs with a linearity of $R^2 = 0.99$. Thus, the limit of detection (LOD) was found 230 mg of CPFA/ kg of milk fat. Whereas the GC-MS method resulted to a LOD of 7.5 mg of CPFA/ kg of milk fat.

Table 1 *Contingency table summarizing the results of the presence of CPFAs in milk samples analysed by [1]H-NMR and GC-MS.*

		Haymilk	Grass silage	Maize silage	Chi-square test*
GC-MS	CPFAs present	0*	80	96	< 0.005
	CPFAs absent	49*	17	3	
NMR	CPFAs present	0*	74	96	< 0.005
	CPFAs absent	49*	23	3	
	Total number of samples	49	97	99	

*Significantly different at $p < 0.05$.

Both methods confirmed the authenticity of Haymilk samples with 100% accuracy (Table 1). Moreover, [1]H-NMR was able to detect the presence of CPFAs at 97% accuracy for milk from maize silage feeding and 76% for grass silage feeding. Overall, the results of [1]H-NMR were in accordance with GC-MS analysis.

3.2 Development of an untargeted 1H-NMR fingerprinting approach to validate the authenticity of Haymilk

According Untargeted 1H-NMR Haymilk fingerprinting analysis have been performed on the 245 milk samples. Multivariate data analysis resulted in a good separation between Haymilk and milk from both grass and maize silage feedings collected in winter. However, discrimination of milk samples collected on summer was challenging due to similar metabolic profiling of the milk samples caused by outdoor grazing of the cows on summer. More powerful discrimination analysis tools are to be used to obtain better results on summer milk samples.

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Ozone technology for sanitization and product quality in the dairy supply chain

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The first activities of the PhD thesis project are described below. The application of low levels of gaseous ozone during Toma Piemontese PDO cheese ripening to control microbial and fungal growth on the crust area was tested. After ozone treatments, cheese spoilage was monitored for 1 month. Every 7 days of ripening, microbiological and technological analyses, and consumer tests on cheese with a minimum of 40 days of ripening, were carried out to evaluate the product quality. Results showed that treatments with low levels of gaseous ozone can significantly affect spoilage microflora, without altering the overall cheese quality.

La tecnologia dell'ozono per la sanitizzazione e la qualità di prodotto nella filiera di produzione casearia

Di seguito sono descritte le prime attività del progetto di tesi di dottorato. È stata testata l'applicazione di bassi livelli di ozono gassoso durante la stagionatura del formaggio Toma Piemontese DOP per controllare la crescita microbica e fungina nell'area della crosta. Dopo i trattamenti con ozono, la crescita microbica e fungina sul formaggio è stata monitorata per 1 mese. Ogni 7 giorni di stagionatura, sono state effettuate analisi microbiologiche e tecnologiche e condotti test sui consumatori su formaggi con minimo 40 giorni di maturazione per valutare la qualità del prodotto. I risultati hanno mostrato che i trattamenti con bassi livelli di ozono gassoso possono influenzare significativamente la crescita della microflora alterante, senza compromettere la qualità globale del formaggio.

Key words: Cheese ripening, ozone technology, product quality, cheese storage, food spoilage, sensory analysis.

1. Introduction

In accordance with the PhD thesis project previously described (Rolle *et al.*, 2022), this poster reports the main results of the first activities concerning:
(A1) the determination of the optimal doses of ozone, contact times and other treatment variables;
(A3) the analysis of the product quality;
(A4) the study of shelf life.

2. Materials and Methods

The batch test involved a control (CTL) and two different samples: gaseous ozone at 400 ppb (0.856 mg m^{-3}), called OZ 400 A, with treatment every other day (8h – overnight) for the entire period of cheese ripening and gaseous ozone at 300 ppb (0.642 mg m^{-3}), called OZ 300 C, all nights (8 h per day) until the end of ripening. Eight cheeses per type of sample were placed in three different rooms (8.0 m^3 each), one with normal atmospheric air for the CTL sample, and the other two rooms connected to the ozone generator (C32-AG; Industrie De Nora Spa, Milan, Italy) via silicone tubes. Rooms were set at 8°C, with 85% relative humidity (RH) (same parameters used by the producer). After 60 days of aging (of which 25 days of cheese ripening by the manufacturer), all samples were stored in the same room (8.0 m^3) set at 8°C for a month to monitor the spoilage microflora growth of cheeses not sold immediately. For weight loss, three cheese samples per treatment were weighed at each sampling time with a technical balance (Adam Equipment Co., Ltd., Milton Keynes). Cheese firmness was evaluated with cylindrical samples by compression test with the Instron Universal Testing Machine (mod. 4301; Instron Inc., Canton, MS, USA) and expressed in N mm^{-1}. The Instron machine was also used to evaluate the cheese chewiness (N) with cube-shaped samples by double compression cycle (Texture Profile Analysis–TPA) (AGRIS). Color evaluation was carried out using a Minolta colorimeter (Minolta C2500; Konica Minolta, Ramsey, NY, USA): chromaticity values L* (lightness), a* (green to red), and b* (blue to yellow) were determined, and color difference CIE 1976 - $\Delta E^* = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{(1/2)}$ - was calculated. For microbiological analysis, three sub-samples were taken from each cheese wheel per type of sample (2.35 cm^2) with a 1 cm diameter round, selecting only the rind. The preparation of samples was made according to ISO 6611 (ISO 6611:2004). For the determination of Total Microbial Count (TMC) and Total Fungal Count (TFC), YEA (Yeast Extract Agar) and PDA (Potato Dextrose Agar) were used, respectively. Plates were incubated at 25 °C for 48h. Data were expressed as the number of colony-forming units (log CFU cm^{-2} of cheese rind). Liking tests were conducted according to ISO 4121 (ISO 4121:2003). Product acceptability for "appearance", "smell", "taste", "aroma", "texture" and "overall acceptability"

on a scale from 1 (extremely bad) to 9 (extremely good) was tested by ten people for each session, who was asked to also indicate flavors perceived on the palate. Data were expressed as the means ± standard error (SE). Analysis of variance (ANOVA) and Tukey's test at the 5% level were carried out, using DSAASTAT tool in EXCEL®.

3. Results and Discussion

3.1 Determination of the ideal parameters for batch test

Optimal doses of ozone, contact times, and other treatment variables were found after carrying out a preliminary test on Toma Piemontese PDO cheese.

3.2 Determination of the effects of gaseous ozone treatments on spoilage microflora and evaluation of qualitative parameters during cheese ripening

All samples gradually lose weight. At the end of ripening, 400 A samples show a lower water loss than CTL (significant difference). For color variation, no significant differences were recorded at any single time between samples. At the end of ripening, all samples appear to have a perceptible color variation compared to the beginning of the batch test (0 days). Firmness data show that treated samples appear to be less consistent than the control (significant difference) at the end of the batch test, probably due to less water loss. However, chewiness analysis shows no significant differences between samples at each sampling time. For TMC, in the long term, ozone-treated samples managed to keep the microbial load at the beginning of the ripening almost stable (no significant difference), compared to that of the CTL sample which, also, significantly grows at the end of ripening by about 0.8 log and 0.9 log compared to the OZ 400 A and OZ 300 C samples. For TFC, treated samples show a significant reduction in the microbial load at the end of ripening of about 1.5 log and 1.4 log for the OZ 400 A and OZ 300 C samples than CTL, respectively. Our results are in agreement with the previous study on pecorino cheese (Grasso *et al.*, 2022). Sensory evaluation shows no significant difference at each session time, except for the appearance score at the end of ripening between CTL and OZ 300 C samples (7,4 vs 6) and for aroma, texture and overall acceptability between the two treated samples which appear to be no different from CTL sample (Table 1), with characteristic nuances of milk and fresh butter perceived more frequently in the treated ones.

Table 1 *Consumer test (mean values ± SE) for the control and the ozone-treated samples (400 and 300 ppb) at 35 days of ripening for Toma Piemontese PDO cheese samples.*

Days	Thesis	Appearance		Smell		Taste		Aroma		Texture		Overall acceptability	
35	CTL	7.4 ± 0.3	a	6.4 ± 0.2	ns	6.5 ± 0.3	ns	6.4 ± 0.3	ab	7.2 ± 0.3	ab	7.1 ± 0.2	ab
	OZ 400 A	6.9 ± 0.2	ab	6.9 ± 0.5	ns	7.3 ± 0.4	ns	7.4 ± 0.3	a	7.4 ± 0.4	a	7.5 ± 0.3	a
	OZ 300 C	6 ± 0.5	b	6 ± 0.4	ns	6.4 ± 0.3	ns	6.3 ± 0.3	b	5.6 ± 0.6	b	6.4 ± 0.3	b

Note: Different lowercase letters for the same column indicate significant differences between cheeses with different treatments ($P < 0.05$).

3.3 Evaluation of microbiological and overall quality during cheese storage after ozone treatments

After the batch test, untreated and treated samples were stored in a room (normal air) for 1 month. Weight loss significantly increases over time for all samples. Also for color variation and firmness no differences were found between samples at the end of storage. For chewiness data, comparing the start and the end of storage for each type of sample, only the CTL sample significantly reduces its chewiness value. TMC decreases over time for all samples, with a significantly lower value for ozone-treated samples. Instead, TFC increases over time for all samples, but data show a significant difference in value from the CTL sample of about 1 log lower for OZ 400 A and 300 C samples. Cheese samples were considered non-different (no significance) by tasters. Typical nuances of milk and fresh butter were confirmed at the end of the storage period. The evaluation of the possible formation of peroxides and other impacts on the nutritional quality of the cheese will be carried out. The first experimental results are promising, so the original PhD thesis project can proceed without any substantial change.

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OLEANOLIC ACID: a Potential Antidiabetic Compound from Aglianico Grape Pomace

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This PhD research project focused on the wine production chain of the Campania region aims at valorizing the production waste by recovering bioactive natural metabolites through sustainable extraction techniques. The Green Chemistry principles will guide the extraction of metabolites, in order to create value-added products in the frame of the circular economy. Red and white grape varieties from the Campania region (Aglianico, Fiano, Greco, and Falanghina) will be studied to identify healthy metabolites and develop optimized extraction protocols. The bioactivity of the extracts will finally be evaluated. The obtained results will suggest the re-use of the identified metabolites in several sectors including the wine, food, pharmaceutical, and nutraceutical industries.

ACIDO OLEANOLICO: un Potenziale Composto Antidiabetico nelle Vinacce di Aglianico

Il progetto di ricerca intende sostenere la filiera vitivinicola della regione Campania attraverso la valorizzazione degli scarti di produzione mediante tecniche di estrazione sostenibili di metaboliti naturali dotati di interessanti proprietà benefiche per la salute dell'uomo. I principi della *Green Chemistry* guideranno l'estrazione dei metaboliti bioattivi e il loro riutilizzo, in modo da creare prodotti dal valore aggiunto nell'ottica dell'economia circolare. Le varietà di uva a bacca rossa e bianca più diffuse in Campania (Aglianico, Fiano, Greco e Falanghina) saranno studiate per identificare i metaboliti di interesse e sviluppare efficienti protocolli di estrazione. La bioattività degli estratti sarà poi valutata. I risultati di tale ricerca suggeriranno un possibile riutilizzo dei metaboliti identificati in campo enologico, alimentare, farmaceutico e nutraceutico.

Key words: Grape pomace valorization, Natural triterpenoids, Red grapes, glucose uptake and mitochondrial activity, C2C12 cells

1. Introduction

Polyphenols have been associated with various bioactivities such as antioxidant, vasodilatory, antithrombotic, cardioprotective, anti-inflammatory, anticancer and antimicrobial ones (Kato-Schwartz et al., 2020; Zhu et al., 2019; Peixoto et al., 2018). Recent studies have shown that grape pomace polyphenols and their polysaccharide conjugates also exhibit antidiabetic effects (Campos et al., 2021). However, it is now evident that the remarkable potential of health-related benefits demonstrated by grape pomace extracts, cannot be attributed solely to the presence of polyphenols. Thus, besides polyphenols, additional metabolites, so far undetected, may be likely occurring. In order to verify such hypothesis, an in-depth chemical analysis of Aglianico (*Vitis vinifera*) red grape pomace was conducted by chromatographic, ^{1}H-NMR and LC-MS/MS techniques.

2. Materials and Methods

In November of 2021, Aglianico grape pomace was collected from the Taurasi area in Italy. A 2-Kg sample was lyophilized to obtain 418 gr of dry material. The dry pomace was extracted twice overnight and under stirring with H_2O:EtOH 2:8 (v/v) at room temperature. The resulting hydroethanolic extract was partitioned against (A) *n*-hexane, (B) Ethyl Acetate (EtOAc), and (C) *n*-butanol. The dry weights of the *n*-hexane, EtOAc, and *n*-butanol extracts were 0.296 gr, 4.95 gr, and 2.496 gr, respectively. The EtOAc extract (B) was further separated through a 80-gr silica Combiflash column (3.0 mL/min flow) connected to a Teledyne Isco CombiFlash Rf flash chromatography system, eluted with the following gradient elution: dichloromethane:EtOAc 1:1 (200 mL; fraction 1), 100% EtOAc (200 mL; fraction 2), EtOAc:MeOH 9:1 (200 mL; fraction 3), EtOAc:MeOH 7:3 (400 mL; fraction 4). Finally, the extracted compounds in each fraction were identified by ^{1}H-NMR, LC-MS/MS, and HPLC. In order to evaluate their biological activities, measurement of mitochondrial activity and glucose uptake were conducted. The mitochondrial activity was measured by using the mitochondrial selective probe MitoTracker CMXRos. The ability of crude extract and fractions to modulate glucose uptake was measured by monitoring the uptake of NBDG, a fluorescent derivative of deoxyglucose covalently bound to the fluorescent chemical nitro blue tetrazolium.

3. Results and Discussion

3.1 Chemical analysis of grape pomace

The most interesting fraction obtained from the Aglianico grape pomace was fraction B. An NMR-based investigation allowed a preliminary identification of triterpenoids, flavan-3-ols, anthocyanins, and polymeric pigments. The occurrence of relatively high quantity of a triterpenoid compound turned out to be the most remarkable datum. Thus, it was purified through chromatographic separation and identified as oleanolic acid by means of NMR spectroscopy and High Resolution ESIMS analysis (Fig.1). The concentration of oleanolic acid in fresh Aglianico pomaces was estimated to hover around 0.45 mg/g.

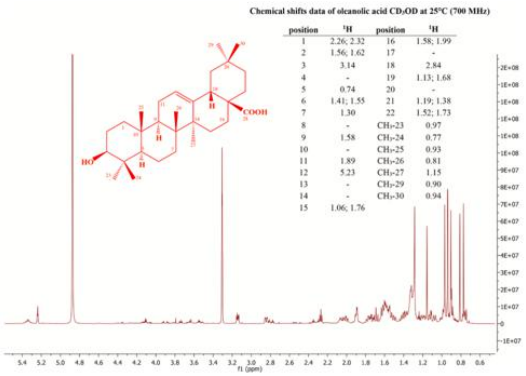

Figure 1 *^{1}H NMR spectrum (CD3OD) of fraction 1 containing Oleanolic Acid as major component*

3.2 Effect of Oleanoic Acid Enriched Fractions on Mitochondrial and Glucose Uptake Activity of In-Vitro Cultured C2C12 Myoblast

The relatively high amount of oleanolic acid recovered from grape pomace provided the opportunity to further investigate its anti-diabetic activity. To investigate the pro-metabolic effect of oleanolic acid, in collaboration with the Department of Pharmacy, its ability to promote mitochondrial activity in C2C12 myoblasts was tested. Oleanolic acid was able to induce mitochondrial activity at the dosage of 1.8 µg/mL, with a dose-dependent potency. Also, its ability to promote glucose uptake via membrane glucose transporters (GLUT) was investigated. Compared to vehicle (DMSO 0.1%), cells treated with oleanolic acid showed an increase in glucose uptake, thus indicating a modulation of glucose uptake through stimulation.

For the first time, an in-depth chemical analysis of Aglianico grape pomace was conducted by means of different chromatographic (column chromatography, UV–Vis HPLC) spectrometric (HRMS, LC-MS/MS) and spectroscopic (NMR) techniques. This investigation disclosed the presence of a number of bioactive compounds, mainly polyphenols. The most relevant outcome of the reported analysis was the isolation of a remarkable amount of oleanolic acid, in the order of 0.45 mg/g of pomace (fresh weight). A promising antidiabetic activity of oleanolic acid was demonstrated. This allowed us to verify our initial hypothesis that additional major bioactive metabolites along with typical grape polyphenols are responsible for the multiple reported bioactivities shown by grape pomace extracts.

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Selection of Next-Generation probiotics from the gut microbiome of subjects with different dietary patterns

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The first activities of the PhD project are described. The aim of the project is to isolate and characterize novel microbial strains from the gut microbiome, for a potential use as Next Generation Probiotics (NGPs). Firstly, nine different culture media and enrichment procedures were tested for the isolation of microbial strains from the human gut, analyzing microbial cells collected from agar plates by 16S rRNA high-throughput amplicon sequencing. Secondly, the four best media were used to isolate microbial strains from the gut microbiome of vegans/vegetarians subjects.

Selezione di probiotici di nuova generazione dal microbioma umano di individui con abitudini alimentari diverse

Le prime attività del progetto di dottorato vengono di seguito descritte. Lo scopo del progetto è isolare e caratterizzare nuovi ceppi microbici dal microbioma intestinale, per un potenziale utilizzo come *Next Generation Probiotics* (NGPs). In primo luogo, sono stati testati nove diversi terreni di coltura e procedure di arricchimento per l'isolamento di ceppi microbici dall'intestino umano, analizzando le cellule microbiche raccolte direttamente dalle piastre mediante sequenziamento ad alto rendimento di ampliconi del gene codificante per l'rRNA 16S. In secondo luogo, sono stati utilizzati i quattro migliori terreni per isolare i ceppi microbici dal microbioma intestinale di soggetti vegani/vegetariani.

Key words: Gut microbiome, Next Generation Probiotics, probiotics, short-chain fatty acids, fibre fermentation

1. Introduction

In accordance with the PhD thesis project previously described, this poster reports the main results of the first two activities concerning:

(A1) selection of suitable media that support the growth of the highest number of putative NGP species; to select strict anaerobes, we tested 9 different culture media and a pre-enrichment step of fecal samples in different broths for 48 h in anaerobic conditions;

(A2) use of the 4 best-performing media for the isolation of microbial strains from 9 vegetarian/vegan donors. Four-hundred twelve microbial isolates were screened and identified by 16S rRNA sequencing.

2. Materials and Methods

We tested nine culture media with different formulations in terms of polysaccharidic source, vitamins, minerals and fatty acids to study the culturable fraction of the gut microbiome. In order to define the best-performing media (in terms of the highest number of putative NGP species growing on them), we collected bulk microbial colonies from agar plates of the different media and sequenced them by 16S rRNA high-throughput amplicon sequencing. The 4 best media were selected for further strain isolation from 9 vegetarian/vegan donors. Colonies were purified using repetitive streaking and the same colony was incubated both in aerobic and anaerobic conditions at 37°C to discard facultative anaerobes. Strains grown only in anaerobic condition were identified by 16S rRNA sequencing. To select strict anaerobes, we also tested a pre-enrichment step of fecal samples in different broths for 48 h in anaerobic conditions. Strains belonging to putative NGP species (as reported in literature, De Filippis et al., 2022) are characterized for the production of beneficial metabolites (e.g., short-chain fatty acids from fibre fermentation, urolithins from ellagic acid, equol from daidzein; Edwards et al., 2017). In addition, we are testing their ability to grow on different carbohydrate sources (e.g., pectin, cellulose, hemicellulose, resistant starch). Short-chain fatty acid will be detected in the growth supernatant using Gas Chromatography coupled to Mass Spectrometry, while urolithins and equol will be detected in supernatant using HPLC analysis.
Promising strains will be finally tested in SHIME (Simulator of Human Intestinal Microbial Ecosystem) to evaluate their effect on health and the ability to modulate the gut microbiome.

3. Results and Discussion

3.1 Selection of suitable culture media

We compared taxonomic composition of the donor fecal sample with that of bulk colonies collected from two plates (10^{-6} and 10^{-7} dilutions) of the 9 media tested, in order to define which media were able to support the growth of the highest diversity of taxa and to reproduce more reliably the microbiota composition of the fecal sample.

Example of microbiota composition from one donor is reported in Figure 1. Results obtained allowed us to select four media that supported the growth of the highest number of putative NGP species, named: PYG; YCFAGSC; Rumen Bacteria medium, *Ruminococcus albus* medium; Chopped meat (Figure 1).

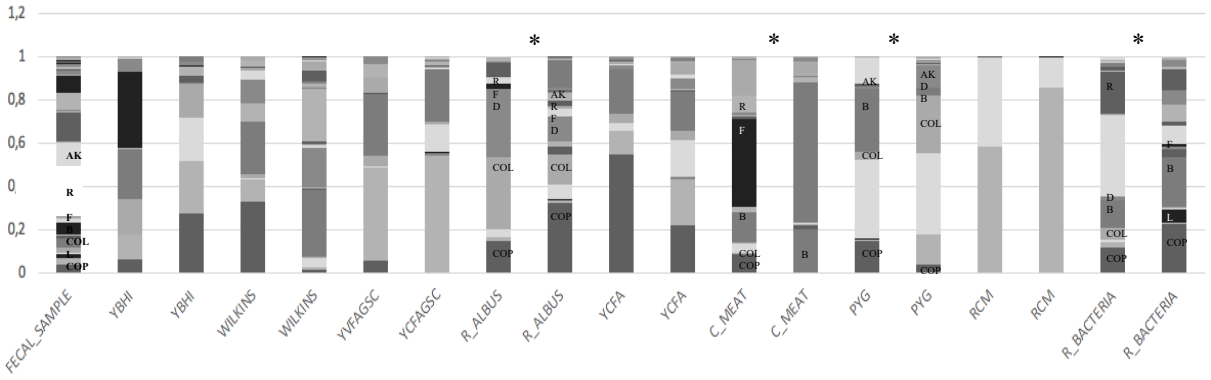

Figure 1 *Bar chart reporting microbiota composition of one donor and of bulk cells collected from two plates of 9 different media tested. The four media selected are circled. Ak = Akkermansia; R = Ruminococcus; F = Faecalibacterium; B = Bacteroides; Col = Collinsella; Cop = Coprococcus; D = Dorea; L = Lachnospiraceae*

3.2 NGP isolation from vegan/vegetarian's gut microbiome

We used the four best performing media (indicated with * in Figure 1) for strain isolation from the fecal samples of 9 vegan/vegetarian donors. We isolated 412 colonies in total, that were purified and screened for growth in aerobic or anaerobic condition. Facultative anaerobes were discarded. After 16S rRNA sequencing and identification, we obtained 42 interesting isolates including the promising NGP candidates *Bacteroides uniformis* and *Bacteroides thetaiotaomicron*, *Collinsella aerofaciens* and *Dorea longicatena* (Table 1). Testing of these strains for the production of beneficial metabolites is on-going.

Table 1 *Putative NGP species identified and culture media where colonies were isolated from. t0, colony isolated from fresh fecal sample; t48, colony isolated from sample enriched in anaerobiosis for 48 hours.*

Isolation media	Taxonomic identification
CHOPPED MEAT t48; PYG + cellobiose + starch t0; RUMEN BACTERIA t48; YCFAGSC t48	*Bacteroides uniformis*
RUMEN BACTERIA t48	*Bacteroides thetaiotaomicron*
YCFAGSC t48	*Bacteroides salyersiae*
PYG + HORSE SERUM t0	*Bacteroides stercoris*
R. ALBUS t48; CHOPPED MEAT t48; YCFAGSC t48	*Parabacteroides distasonis*
YCFAGSC t48; YCFAGSC t0; R. ALBUS t0; PYG + HORSE SERUM t48; R. ALBUS t0; R. ALBUS t48	*Faecalicatena contorta*
PYG + HORSE SERUM t0; PYG + HORSE SERUM t48; YCFAGSC t0	*Collinsella aerofaciens*
PYG + cellobiose + starch t0	*Dorea longicatena*
YCFAGSC t0	*Pseudoruminococcus massiliensis*

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Development of strategies for the adaptation of the livestock sector to the new climate regime with machine learning and artificial intelligence methods

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The first activity was concluded with the identification by ML (Machine Learning) methods of a narrower range of wavelengths in the near infrared for the identification of DM (Dry matter), aNDF (neutral detergent fibre); ADF (acid detergent fibre) and ADL (acid detergent lignin). The second activity is based on the study of metagenomics from the faeces of dairy buffaloes to identify correlations with nutrition and its digestibility.

Sviluppo di strategie per l'adattamento del settore zootecnico al nuovo regime climatico con metodiche di machine learning ed intelligenza artificiale

La prima attività si è conclusa con l'identificazione, tramite metodi ML (Machine Learning), di un intervallo più ristretto di lunghezze d'onda nel vicino infrarosso per l'identificazione di parametri quali: Sostanza secca (DM), fibra neutro detersa (aNDF), fibra acido detersa (ADF), e lignina (ADL). La seconda attività si basa sullo studio della metagenomica dalle feci di bufale da latte al fine di individuare delle correlazioni con l'alimentazione e la digeribilità di questa.

Key words: precision feeding; feaces; microbiome; buffalo**.**

1. Introduction

In accordance with the PhD thesis project previously described (Evangelista, 2022), in this poster reports the main results of the second activity concerning:

(A) The characterization of metagenomic profiles of different buffalo farms and the influencing factors on the microbiome in feaces

2. Materials and Methods

Faecal samples were collected in 10 dairy buffalo herds representative of the Amaseno valley, in the Lazio region. The samples were collected from each company once a month, from June to November 2022 for a total of 6 samplings. The faeces were collected approximately 2-3 hours after the distribution of the morning feed on a representative sample of animals (about 8-10 buffaloes per farm) directly from the rectal ampoule. In addition, samples of TMR (Total Mixed Ration) and bulk milk were collected for each farm. A total of 60 TMR, 60 of faeces, and 60 bulk milks were collected and analysed.
The analyses conducted on TMR, and faeces were: Dry matter (DM) was measured after oven drying at 65°C to constant weight. Then, TMR and feaces samples were ground through a mill (Retsch Müller, Germany) to pass a 1 mm screen and then sealed polyethylene containers were used to store prepared samples. Samples were analysed for crude protein (AOAC, 2005), ash (AOAC, 1990), ethereal extract (AOAC, 2000), and starch (AOAC, 2005) using a K-TSTA assay kit (Megazyme International, Bray, Ireland), and neutral detergent fibre (aNDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) were analysed using an Ankom200 Fiber Analyzer (Ankom Technology, Macedon, NY) according to Van Soest et al. (1991). Furthermore, the pH was determined on the faeces by means of a portable pH-meter ("XS Tester", Giorgio Bormac, Italy). Chemical data are reported as percentages on a dry matter basis. MIlk analyzes were conducted by Experimental Zooprophylactic Institute of Rome.
DNA extraction for sequencing according to the NGS (next generation sequences) technique, the kit was used: Quick-DNATM Faecal/Soil Microbe Miniprep Kit (Zymo Research Corporation, USA).

3. Results and Discussion

3.1 Determination of chemical composition of feceas, TMR and bulk milk

Table 1 shows the chemical composition of buffalo faeces (Mean ± SD).

Table 1 Chemical composition of faeces.

	DM (%)	Ash (%)	CP (%)	EE (%)	aNDF (%)	ADF (%)	ADL (%)	Starch (%)	pH
Means (±SD)	13.76 (±1.39)	12.16 (±1.26)	14.12 (±1.27)	1.29 (±0.31)	56.50 (±3.84)	43.88 (±3.25)	14.78 (±2.24)	1.71 (±1.19)	6.34 (±0.22)
Max	19.14	15.55	17.96	2.43	64.24	50.51	20.40	8.34	6.73
Min.	11.34	9.67	11.16	0.83	43.38	35.11	10.49	0.78	5.73

DM: dry matter; ash; crude protein; ethereal extract; aNDF (neutral detergent fibre); ADF (acid detergent fibre); ADL (acid detergent lignin); and starch are on DM basis.

Table 2 shows the results relating to the chemical-physical composition of the TMR (Mean ±SD).

Table 2 Chemical-physical composition of the TMR.

	DM (%)	Ash (%)	CP (%)	EE (%)	aNDF (%)	ADF (%)	ADL (%)	Starch (%)	Upper (%)	Middle (%)	Lower (%)	Bottom (%)
Means (±SD)	52.12 (±6.47)	6.80 (±0.88)	11.87 (±1.98)	2.48 (±0.43)	46.61 (±5.32)	31.10 (±3.80)	6.13 (±0.95)	13.66 (±2.69)	21.64 (±15.45)	25.16 (±9.89)	28.46 (±8.46)	24.82 (±6.06)
Max	67.32	10.76	15.48	3.78	57.70	43.34	8.50	17.80	61.20	43.90	44.90	37.30
Min.	40.98	5.23	6.13	1.70	36.79	23.94	3.20	5.83	1.00	6.30	13.60	12.70

DM: dry matter; ash; crude protein; ethereal extract; aNDF (neutral detergent fibre); ADF (acid detergent fibre); ADL (acid detergent lignin); and starch are on DM basis; Upper = % of ration retained by a sieve with holes of 19 mm; Middle = % of the ration retained by a sieve of 8 mm; Lower = % of the ration retained by a sieve of 4 mm; bottom =Bottom of ration with dimensions <4 mm.

Table 3 shows the results relating to the chemical composition of the bulk milk (Mean ± SD).

Table 3 Chemical composition of buffalo bulk milk.

	Fat (%)	Protein (%)	Lactose (%)	Casein (%)	Urea (mg/dl)	pH	Acidity (°SH/100)	SCC (*1000/ml)	RCT (min.)	K_{20} (min.)	A_{30} (min.)
Means (±SD)	8.31 (±0.56)	4.73 (±0.16)	4.57 (±0.06)	3.86 (±0.18)	41.81 (±5.45)	6.76 (±0.10)	7.83 (±0.58)	265.50 (±155.13)	21.23 (±5.25)	4.63 (±2.84)	23.12 (±10.27)
Max	9.67	5.23	4.68	4.32	54.2	6.94	9.98	699	36.37	21	45.10
Min.	7.09	4.46	4.38	3.40	29.50	6.19	6.50	78	4.37	2.30	2.90

SCC: somatic cell count; RCT: rennet coagulation time, min; k_{20}: curd firming time, min; a_{30}: curd firmness, mm.

DNA extractions from faeces samples have just been completed. The DNA will be sequenced in the coming months.

The results on the composition of the ration and faeces show a wide variability mainly due to the different feed management adopted by the 10 farms. From the results of the NGS analysis we therefore expect to have a microbial variability within the faeces. In this way we will try to understand how diet influences the microbial composition of faeces.

3.2 Formulation of the research program for the following year

The next phase involves the analysis and study of the results obtained from the NGS on the faeces microbiome. The following step involves an in vivo study to test the ability of a supplement based on cellulolytic bacteria to improve the digestive efficiency of the fiber in dairy buffaloes.

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The valorization of sustainable food matrices for the development of new food ingredients and products

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Lemon peel flour (LPF), produced from a by-product of limoncello production, was used as ingredient in the formulation of different meat-analogues. A soybean burger and soybean strips were taken as control samples. A proportion of 2-4% of LPF was added to the base recipe of the burger to replace completely the fibers and partially the fat. For the soybean strips, LPF was added at 2% as part of the coating. The digestibility of the meat-analogues was assessed by in vitro digestion. The addition of LPF showed promising results as replacer of commercial fibers and fats when added at 2%.

La valorizzazione di matrici alimentari sostenibili per lo sviluppo di nuovi ingredienti e prodotti alimentari

La farina di bucce di limone (FBL), ricavata da un sottoprodotto della produzione di limoncello, è stata utilizzata come ingrediente nella formulazione di analoghi della carne. Un burger di soia e straccetti di soia sono stati presi come campioni di controllo. La FBL è stata aggiunta (2%; 4%) al burger per sostituire completamente le fibre e parzialmente il grasso; per gli straccetti di soia, è stata aggiunta al 2% come parte del condimento. Si è studiata la digeribilità dei prodotti mediante digestione in-vitro. La FBL ha dato risultati promettenti come sostituto delle fibre e dei grassi, se aggiunta al 2%.

Key words: Citrus limon wastes, soybean burger, meat analogues, in vitro digestion, fat replacer.

1. Introduction

In accordance with the PhD thesis project, this poster reports the main results of the first activities concerning:
- the use of lemon peel flour as an ingredient in the formulation of a meat analogue;
- the digestibility of the meat analogues.

2. Materials and Methods

Lemon peels residual from the production of "limoncello" were freeze-dried and ground to obtain flour with particle size < 1 mm. The lemon peel flour (LPF) was used as an ingredient in two base meat analogues recipes. The soybean burger (SB) recipe ingredients were: soy protein concentrate, vegetable oils (sunflower, coconut), natural flavors, soy protein isolate, methylcellulose, starch (potato, tapioca), dried yeast, vegetable fiber (pea and bamboo), burnt sugar, salt. LPF was introduced at different percentages, 2% (LPF 2% SB) and 4% (LPF 4% SB) by fully replacing the fiber and partially the fat respectively at 12% and 36%.
The soybean strips (SS) recipe ingredients were: soy protein extract (water, soy protein 35%, vegetable fiber), sunflower seed oil, natural flavors, yeast extracts and salt. LPF was added (2%) in the coating of the soybean strips (LPF 2% SS). All the samples were produced in an industrial plant. The main ingredients contributor for the protein and carbohydrates were not changed in the formulation do to have comparable protein and carbohydrates content cross the evaluated samples. The protein content was determined by the Kjeldahl method (N × 5.71). The different meat analogues were digested according to the INFOGEST in vitro digestion protocol (Brodkorb et al., 2019). After digestion, glucose release was assessed by enzymatic assay for D-Glucose Enzytec™, total protein digestibility was determined after acid hydrolysis by total amino groups (o-phthalaldehyde method).

3. Results and Discussion

3.1 Nutritional composition

The nutritional composition of soybean burger and soybean strips used as control samples is reported in Table 1 and Table 2. The protein content of the samples was confirmed by the Kjeldahl method.

Table 2 *Nutritional table of soybean burger.*

Nutritional table	Serving size (100 g)
kJ/kcal	915/219
Total fat	12%
saturated fat	5,4%
Total carbohydrates	13%
sugar	0%
Protein	14%
NaCl	1,5%

Table 1 *Nutritional table of soybean strips.*

Nutritional table	Serving size (100 g)
kJ/kcal	805/196
Total fat	7,8%
saturated fat	0,9%
Total carbohydrates	5,4%
sugar	0,4%
Protein	24%
NaCl	1,9%

3.2 Carbohydrates digestibility

Free glucose release at the end of the duodenal digestion phase (120 min) was quantified, being a useful information to understand the effect of the lemon fibre on the release of glucose. For the burgers (SB, LPF 2% SB and LPF 4% SB) the presence of LPF resulted in a lower release of glucose which was proportional to the increasing amount of LPF. As shown in Figure 1, the quantity of free glucose in the three samples was found significantly different with a p value < 0.01.

Figure 1 *Effect of LPF addition to the formulation of soybean burger on the release of glucose through the digestion, D-Glucose (mg/5g sample).*

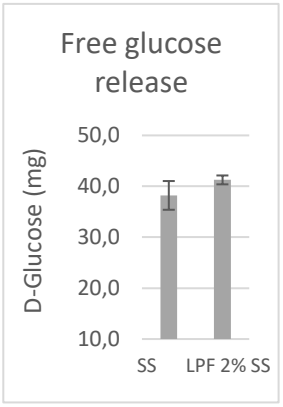

Figure 2 *D-Glucose (mg/5g sample) in soybean strips.*

For soybean strips the statistical analysis showed no differences in terms of glucose release considering a significance level of 0.05 as reported in Figure 2.

3.2 Total protein digestibility

Total protein digestibility was calculated using the following equation:

$$\text{Digestibility [\%]} = \frac{Fs - Cs}{Food} \times 100 \quad (1)$$

Fs=NOPA of the supernatant of digested food (mg)
Cs=NOPA of the supernatant of the enzyme blank (mg)
Food=NOPA of the food as is (mg)

Previous *in vivo* and *in vitro* studies demonstrated high digestibility scores both in terms of total digestibility of the proteins and digestible essential amino acids for soybean protein isolates, ranging from 84 to 90.6% (Van den Berg et al., 2022).

The protein digestibility was found to be 70% and as showed in the Figure 3 is concordant among the burger samples. For soybean strips, the digestibility was 68%. This lower digestibility can be attributed either to the structure of the food matrix or to the changes the proteins undergo during the extrusion process. These promising data support the introduction of lemon wastes to improve the nutritional quality of plant-based meat analogues.

These data suggest that LPF could be a valid replacer of commercial fiber and fat. However, further sensorial and rheological analysis are needed.

The experimental results are promising for the prosecution of the designed PhD thesis project and for proceeding with the scheduled analysis workflow.

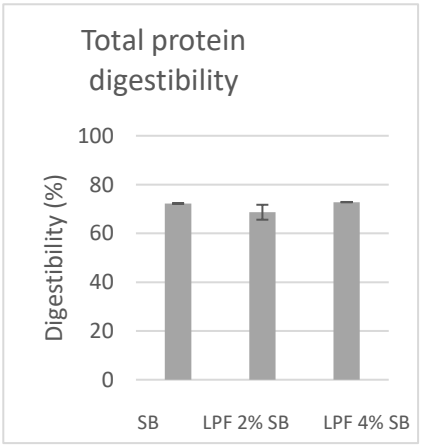

Figure 3 *Total protein digestibility of soybean burgers.*

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Applications of Cold Atmospheric Plasma as *Green* Technology for Food Shelf-life Extension

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The research of this PhD thesis here presented is aimed to investigate the application of plasma activated water (PAW) in starch modification, to increase the knowledge of its effects on different starch matrices during processing. Potato, normal maize and waxy maize starches were treated with PAW, whereupon an increase in most pasting, thermal, rheological and gel hardness parameters was observed for potato and normal maize starches, while the opposite result was observed for waxy maize. The results obtained in this study highlighted that PAW is a promising novel alternative method to modify the properties of starch.

Applicazioni del atmosferica plasma freddo come trattamento *green* per il prolungamento della shelf-life degli alimenti

La ricerca qui presentata parte del progetto di dottorato ha lo scopo di indagare le applicazioni dell'acqua attivata al plasma (PAW) sulla modifica di amido, al fine di aumentare la conoscenza dei suoi effetti su diverse matrici amidacee durante la lavorazione. Amido di patata, di mais e di mais 'ceroso' (*waxy*) sono stati trattati con PAW. Tale trattamento ha causato un aumento della maggior parte dei parametri termici e reologici investigati per l'amido di patata e di mais, mentre per il mais *waxy* gli effetti sono stati tendenzialmente opposti. I risultati ottenuti in questo studio hanno evidenziato come il trattamento con PAW risulti un potenziale metodo alternativo per modificare le proprietà dell'amido.

Keywords: Starch, cold plasma, functionalization, modification

1. Introduction

In accordance with the PhD thesis project previously described (Gebremedhin, 2022), this poster reports the main results of the first two activities concerning:
(A2.1) Effects of Plasma Activated Water (PAW) on rheological, thermal and pasting properties of potato, normal maize and waxy maize starches;
(A2.2) The effect of PAW on chemical and functional properties of potato, normal maize and waxy maize.

2. Materials and Methods

Potato (P), normal maize (NM), and waxy maize (WM) were obtained from Padovana Macinazione, Padova, Italy. The plasma-activated water (PAW) equipment was provided by AlmaPlasma Srl (Bologna, Italy); PAW was generated by exposing the plasma discharge using corona source in 500 mL of distilled water for 1 min, at 15kV and 5 kHz. The average discharge power (P) was measured by connecting a high-voltage probe and a current probe to a digital oscilloscope to measure voltage (V) and current (I) during plasma treatment. The Hydrogen Peroxide Assay Kit and the Nitrate/Nitrite colorimetric assay were used for the measurement of H_2O_2 and NO_2^- concentrations in PAW respectively, using a microplate reader. Moreover, the pH was evaluated using a pH Meter. After PAW generation, starches were mixed with PAW in a 1:2 (w/v) (starch: PAW) ratio, and stirred continuously for 20 min. For each starch, a sample mixed with distilled water was considered as control. Native and PAW-treated starches were oven dried at 40 °C overnight, grounded, sieved, and stored for further analysis. Pasting properties, gel hydration properties, gel hardness, and flow behavior were analyzed. Significant differences between the means of the parameters of native and treated samples (within the same starch) were evaluated through the Student-t test (p-level > 0.05).

3. Results and Discussion

Plasma-activated water (PAW) was generated with a corona discharge at 15kV and 5 kHz for 1 min, and was applied to test its potential ability to modify the structure and functionality of potato, normal maize, and waxy

maize starches. After the PAW was generated, it was mixed with the starches (1:2; starch: PAW), for 20 min. Starches were then evaluated for various characteristics (rheological, thermal, and functional properties) and FTIR. The average discharge power was ~167.12W, and the concentrations of the main long-lived species highlighted the production of hydrogen peroxide (1.18 mg/l), nitrites (13.98 mg/l), and a reduction of pH from 6.12 (distilled water) to 3.48. The pasting properties, gel hydration, gel hardness, and viscosity of the properties of native and PAW treated potato (P), normal (NM), and waxy maize (WM) are shown in Tables 1 and 2, respectively. The results for the pasting properties for the P sample, and statistical analysis revealed that PAW-treated samples were characterized by significantly higher values for all parameters compared to the native one, except for the setback viscosity (SBV). Similarly, except for pasting temperature (PT), other values were increased for the NM sample, although differences were found significant only for breakdown viscosity (BV). On the other hand, PAW treatment significantly ($P< 0.05$) reduced the value of peak viscosity (PV), holding strength viscosity (HSV), SBV, and final viscosity (FV) in WM compared to the native starch.

Table 1 *Pasting parameters of native and PAW-treated potato (P), normal maize (NM), and waxy maize (WM) starch*

Starches		PT(°C)	PV(Pa.s)	HSV(Pa.s)	BV(Pa.s)	SBV(Pa.s)	FV(Pa.s)
P	Native	64.19±0.68^{b}	12.34±0.19^{b}	2.73±0.01^{b}	9.61±0.20^{b}	1.73±0.14	4.46±0.14^{b}
	PAW	65.05±0.60^{a}	15.78±0.9^{a}	3.26±0.10^{a}	12.52±0.03^{a}	1.9±0.07	5.16±0.03^{a}
NM	Native	75.54±0.95^{a}	3.67±0.19	1.78±0.04	1.88±0.15^{b}	1.78±0.13	3.57±0.17
	PAW	71.72±0.22^{b}	3.85±0.17	1.83±0.02	2.02±0.15^{a}	1.92±0.14	3.75±0.16
WM	Native	70.72±0.20	4.25±0.12^{a}	1.37±0.03^{a}	2.87±0.10	0.55±0.02^{a}	1.93±0.04^{a}
	PAW	70.63±0.25	3.22±0.18^{b}	0.46±0.07^{b}	2.75±0.13	0.24±0.03^{b}	0.71±0.10^{b}

The effect of PAW treatment on the gel hydration properties namely Swelling Power (SP) and Water Solubility Index (WSI) at 90°C and on the gel hardness are reported in Table 2. PAW treatment significantly ($p<0.05$) changed the SP value of starches, from 11.98±1.80, 15.07±0.91 and 12.98±0.23 for native WM, NM, and P, to 22.49±6.86, 16.52±0.23 and 18.07±0.61 in PAW treated samples, respectively. The WSI of starches treated with PAW was higher than the native ones, but in a significant only for NM and WM. This change may be due to the leaching out of starch fragments caused by PAW reactive particles (Aaliya et al., 2022). Concerning gel hardness, similarly to other parameters, a different behavior was observed in relation to the types of starch. increased hardness was detected for P and NM starches after PAW treatment, while PAW applied on WM starch caused a decrease. In general, even if the consistency coefficient (k) and the flow index (n) were different for the three analyzed starches, the flow behaviors of all three samples were typical of pseudo-plastic fluids (shear thinning region) because of $n<1$. As depicted in Table 2, the correlation coefficients (R^2) was 0.95–0.99 for all samples, indicating a satisfying fit. PAW treatment resulted in a substantial and significant ($P<0.05$) increase in the viscosity value (k) and a decrease of the flow behavior index (n) between native and PAW-treated samples for P and NM, probably because of the re-association of the starch chain by the reduction of hydroxyl groups in the starch structure due to the effect of the reactive species in PAW. The enhancement of the interaction among starch particles caused high resistance to flow (Chou et al., 2023). On the contrary, the viscosity of WM starch was significantly decreased by PAW, probably promoted by the depolymerization of starch chains.

Table 2 *Influences of PAW treatment on gel hydration, gel hardness, and flow behavior of native and PAW treated potato (P), normal maize (NM), and waxy maize (WM) starch*

Starches		SP (g/g)	WSI (g/g)	Hardness (N)	k (Pa.s^{n})	n	R^2
P	Native	12.98±0.23^{b}	6.23±0.53	4.26±0.51^{b}	47.91±0.12^{b}	0.50±0.03^{a}	0.99
	PAW	18.07±0.61^{a}	6.45±0.37	6.90±0.44^{a}	88.74±1.14^{a}	0.44±0.02^{b}	0.99
NM	Native	15.07±0.91^{b}	10.85±1.29^{b}	4.67±0.10^{b}	11.7±0.64^{b}	0.46±0.01^{a}	0.99
	PAW	16.52±0.23^{a}	12.78±1.74^{a}	5.25±0.23^{a}	58.56±2.42^{a}	0.24±0.01^{b}	0.95
WM	Native	11.98±1.80^{b}	22.52±3.86^{b}	0.57±0.05^{a}	6.85±0.59^{a}	0.38±0.01	0.99
	PAW	22.49±6.86^{a}	44.88±2.85^{a}	0.44±0.02^{b}	4.63±0.18^{b}	0.39±0.01	0.99

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Integrated Green Strategies for the Management, Recovery, and Recycling of Waste in a Dairy Factory

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A casein-based biofilm with tailored mechanical, thermal and vapour-barrier properties was developed using Sodium caseinate (NaCas). Casein was obtained by isoelectric precipitation from expired pasteurized milk with subsequent washing, solubilization with alkali and lyophilisation. A commercial NaCas was used as control. The two NaCas were used to prepare film forming solutions including glycerol. The casting method on silicon molds was used for film production followed by drying under controlled conditions. Structure of films was characterized by confocal laser scanning microscopy with image analysis, while FT-IR was used to investigate the changes at the protein network. Finally, preliminary mechanical tests were carried out.

Strategie Green Integrate per la Gestione, il Recupero e il Riciclo di Sottoprodotti nell'Industria Lattiero-casearia

È stato sviluppato un biofilm a base di caseina con adeguate proprietà meccaniche, termiche e di barriera al vapore, utilizzando sodio caseinato (NaCas). La caseina è stata ottenuta per precipitazione isoelettrica da latte pastorizzato scaduto, seguita da lavaggio, solubilizzazione con alcali e liofilizzazione. NaCas commerciale è stato utilizzato come controllo. Le due polveri sono state utilizzate per preparare soluzioni, comprensive di glicerolo, per la formazione di film. I film sono stati prodotti mediante casting su stampi in silicone e successiva essiccazione in condizioni controllate. La struttura dei film ottenuti dai due tipi di NaCas è stata caratterizzata mediante microscopia a scansione laser confocale con analisi delle immagini, mentre la tecnica FT-IR è stata utilizzata per indagare le modifiche del network proteico. Infine, sono stati effettuati test meccanici preliminari.

Key words: Milk, casein, biofilm, sustainability, protein network, fat globules.

1. Introduction

Unsold pasteurized milk that reaches the expire date is no longer suitable for human consumption and it is downgraded to "Special Category III waste". Casein has, however, unique technological properties and can be easily recovered from milk by isoelectric precipitation and addition of alkali to obtain soluble caseinate (NaCas). Recently, the use of NaCas for preparing biofilms has been proposed [1]. Like other protein-based films, casein films have positive characteristics, including good mechanical and gas barrier properties but, on the other hand, some weaknesses like high water-vapor permeability (WVP) [2]. Additives can be used in film formulation, such as glycerol, to improve the flexibility of the protein network, while hydrophobic components like beeswax or oils can improve WVP. Furthermore, formation of covalent crosslinks in the casein network through treatment by microbial transglutaminase or tannic acid may help as well [1]. Solution casting is the most used technique to obtain films at lab scale [3]. Based on this background, this PhD project focuses on the production of a casein film having suitable performances for various non-food uses, in order to give a novel high-valued destination to milk wasted from the food chain. Possible approaches for casein modification and additives that can be added to the formulation for the improvement of the film's characteristics will be studied. Finally, a deep chemical, mechanical and thermal characterisation of the film will be done to find the most appropriate applications in real situations.

2. Materials and Methods

2.1 NaCas production and characterization: Casein was isolated from expired (7-day old) pasteurized milk by isoelectric precipitation, washing for lactose and fat removal, alkali (NaOH) neutralisation to obtain soluble NaCas (NaCas A') and lyophilisation. A centrifugation step at 40 °C was applied to NaCas A', with the aim of preparing a caseinate with lower fat content (NaCas A). Finally, a commercial NaCas was used as a control sample (NaCas C). The gross composition of the three NaCas was evaluated using the ISO standard methods.
2.2 Production and development of the biofilms: NaCas was solubilized in water (10% w/v) and heat treated at 90 °C for 30 min under stirring. Glycerol (33% w/w on protein content) was added, the solution was stirred for another 10 min and then cooled to room temperature. Film-forming solution was poured on silicon moulds and let dry in a climatic chamber at 23 °C and 55% RH for 48 hours. After drying, the film was peeled off and maintained at ambient conditions. This procedure was used to produce films from NaCas A', A, and C.
2.3 Characterization of the biofilms:
- *Confocal Laser Scanning Microscopy:* CLSM was carried out on films to evaluate the behaviour of fat within

the film matrix. Fat structures were characterized by image analysis (ImageJ software).
- *Fourier Transform Infra-Red Spectroscopy:* FT-IR analysis was performed using attenuated total reflectance (ATR. Measurements were carried out with a resolution of 4 cm^{-1} and 64 scans. This analysis had the aim to investigate the possible changes that have occurred to the film structure during production.
- *Mechanical Properties:* analyses were performed using a texture analyzer, following the method ASTM D882, with a load cell of 50 kg. Only film C and Film A have been analyzed so far.

3. Preliminary Results and Discussion

3.1 Characterisation of NaCas samples

The additional fat removal step allowed to obtain NaCas A with a lower fat content (23.3% vs 30.9%).

Table 1 *Chemical composition of NaCas samples.*

	NaCas C	NaCas A'	NaCas A
Protein (g/100g)	96	60.7 ± 0.6	66.1 ± 0.8
Fat (g/100g)	~ 0	30.9 ± 0.1	23.3 ± 0.2
Moisture (g/100g)	3	5.43 ± 0.1	ND

3.2 Confocal Microscopy and Image analysis

Film structure was investigated by CLSM (Fig. 1). Film A' showed large heterogeneity in size and shape of fat structures having an average diameter of 1.35 µm and few structures up to 60 µm. Fat was also unevenly allocated within the film. After the additional fat removal step, fat organization in Film A improved, with structures similar in size and more regularly distributed (Fig. 1b, 1d). Fat organisation was completely different in Film C, where small fat structures of 0.60 µm in diameter and higher circularity than in the other films were seen. Film C also showed insolubilized protein particles originating from NaCas C (Fig. 1c arrows) and possibly caused by the industrial manufacturing process. Since all films were produced from NaCas containing homogenized fat (particle size ~1 µm), large fat structures in Films A' and A derive from fat coalescence during film making.

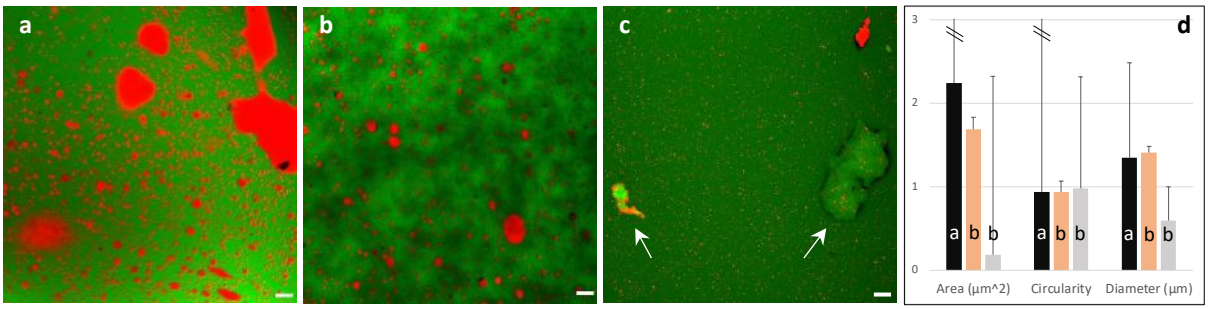

Figure 1 *CLSM images of protein (green) and fat (red) in Film A' (a), Film A (b) and Film C (c). Bar in panels a, b, c, is 10 µm in length. Panel d) shows the results of image analysis related to image a) (black column), image b (orange column) and image c (grey column).*

3.3 FT-IR

Functional groups of the films are observed with FT-IR analysis. Fig. 2 shows spectra recorded for Film C and A. Typical casein bands are in the range 3400-2800 cm^{-1} for -OH and -NH groups, while bands in the range 1700-1500 cm^{-1} are related to amide I° and II° groups. Significant changes can be attributed to the bands related to fat functional groups, confirming CLSM hints. The bands at 2930-2900 cm^{-1} can be attributed to stretching of C-H groups, bands at 1167-1050 cm^{-1} to stretching of C-O groups, and bands at 1740-1735 cm^{-1} to the stretching of C=O groups. These bands have higher intensity in Film A than in Film C due to the higher free fat content.

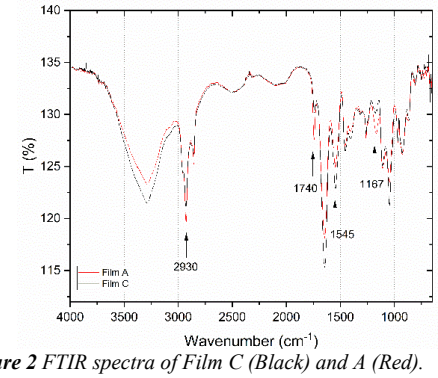

Figure 2 *FTIR spectra of Film C (Black) and A (Red).*

3.4 Mechanical Properties

Material integrity under stress condition that could occur during lifespan of the film can be evaluated with tensile strength (TS) and elongation at break (EAB). TS was 5.9 ±0.8 MPa for Film A and 7.1 ±1.0 MPa for Film C, EAB was 120.8 ±19.2% for Film A and 102.1 ±20.5% for Film C. The lower TS of Film A ($p < 0.05$) is likely due to a weaker casein network caused by the higher presence of fat structures, compared to Film C. The same reason can explain higher EAB in Film A ($p < 0.05$), where fat structures make the matrix of the film more extensible.

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Evolution of green and black kombucha tea microbial consortia composition and *in vitro* bioactivity during six successive monthly preparations

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Preliminary results of the main activities investigated during the two years of PhD are presented. Kombucha is traditionally produced from the fermentation of green or black tea by a cellulosic biofilm known as SCOBY (Symbiotic Culture of Bacteria and Yeasts), which consists of a symbiosis of acetic bacteria, lactic acid bacteria, and yeasts. The aim of the project is to study the evolution of the microbial population in both black and green kombucha drinks during a consecutive period of six months production, with monthly intervals. Furthermore, thestudy evaluates correlations among different consecutive production of kombucha beverages and related bioactivities, using *in vitro* tests.

Evoluzione della composizione microbica di kombucha verde e nero e bioattività *in vitro* di sei mesi consecutivi

Di seguito sono presentati i risultati preliminari delle principali attività svolte durante i due anni di dottorato. Il kombucha è tradizionalmente prodotto dalla fermentazione di tè verde o nero mediante un biofilm cellulosico, noto come SCOBY (Symbiotic Culture of Bacteria and Yeasts), che consiste in una simbiosi di batteri acetici, lattici e lieviti. Lo scopo del progetto è determinare l'evoluzione della composizione microbica di kombucha neroe verde durante un periodo consecutivo di sei mesi di produzione fatta a cadenza mensile. Inoltre, lo studio valutale correlazioni tra diverse produzioni consecutive di kombucha e le relative bioattività, utilizzando test *in vitro*.

Key words: Kombucha, SCOBY (Symbiotic Culture of Bacteria and Yeasts), fermentation.

1. Introduction

In accordance with the PhD thesis project, the main results of the activities are reported:

- production of black and green kombucha tea over six consecutive months;
- characterization of green and black kombucha at the end of fermentation time trough microbiologicalanalyses and pH measurements.

2. Materials and Methods

For beverage production, two sets of kombucha (green and black) were made each month during the period October 2022 – April 2023. Kombucha was produced from tea leaves according to the methodology used by Cardoso et al. (2020). Firstly, 50 g/L of sucrose were added to 1 L of sterile water. Then, for the infusion stage, black tea (Darjeeling Gielle FTGFOP1 Second Flush) and green tea (Lung Ching) were added at a concentration of 12 g/L in the water at 95 °C for 4 min and 75 °C for 1 min, respectively. After infusion, the beverage was strained through a cotton gauze and poured into sterile glass jars of 18 cm height and 10 cm diameter. The tea waskept in an ice bath to reach room temperature quickly. Then, 3% (w/v) of SCOBY (Enziquímica, Gravataí-RS, Brazil) and 100 mL/L of a previously produced kombucha batch were added (de Noronha et al., 2022). The openingof the jars was covered with a clean cotton cloth and fermentation was carried out in the dark, at 25 ± 2 °C for 5 days for green kombucha and 7 days for black kombucha. After fermentation, kombucha was used for microbiological analyses and pH measurement. The SCOBY was collected with a sterile pinzel and stored in 30 mL of kombucha in a closed tube at 5 °C for future analysis. Kombucha samples were transferred to microtubes (2 mL aliquots), centrifuged at 15,000 rpm for 15 min and stored at - 20 °C until further analysis. The pH of greenand black kombucha after fermentation was measured using a bench pH-meter (Hanna Instruments, USA). Microbiological counts were done using the spread plate technique performed in triplicate. The mesophilic aerobiccount was determined on Plate Count Agar (PCA), while Potato Dextrose Agar (PDA) was used for the yeasts count. Acetic acid bacteria (AAB) were analysed on Glucose Yeast Carbonate agar (glucose 50 g/L, yeast extract10 g/L, calcium carbonate 5 g/L and agar 20 g/L), while lactic acid bacteria (LAB) were counted on Man Rogosa Sharpe (MRS) agar. Plates were incubated at 30 °C for 3 days under aerobic conditions. All the experiments wereperformed in triplicate. Data were analyzed using a one-way analysis of variance (ANOVA). Tukey's test was used as a post-hoc analysis by the GraphPad Prism software (version 7, GraphPad Software, Inc., San Diego, CA,USA). Results were considered significantly different for p values lower than 0.05.

3. Results and Discussion

The first kombucha production (November 2022 for black tea and October 2022 for green tea) showed microbial counts significantly different from the following months, except for yeasts and AAB in black kombucha. During the successive months the average of bacterial and yeasts count showed a reduction in both green and black kombucha over time, largely detected for January. Although production conditions were maintained constant, microbial counts tended to decrease during the first 3 months and then showed an increase towards the first monthvalues. Increase of LAB in green kombucha was particularly relevant. The pH was measured at the beginning (ranging between 4 and 4,5) and at the end of fermentation (Tab. 1).

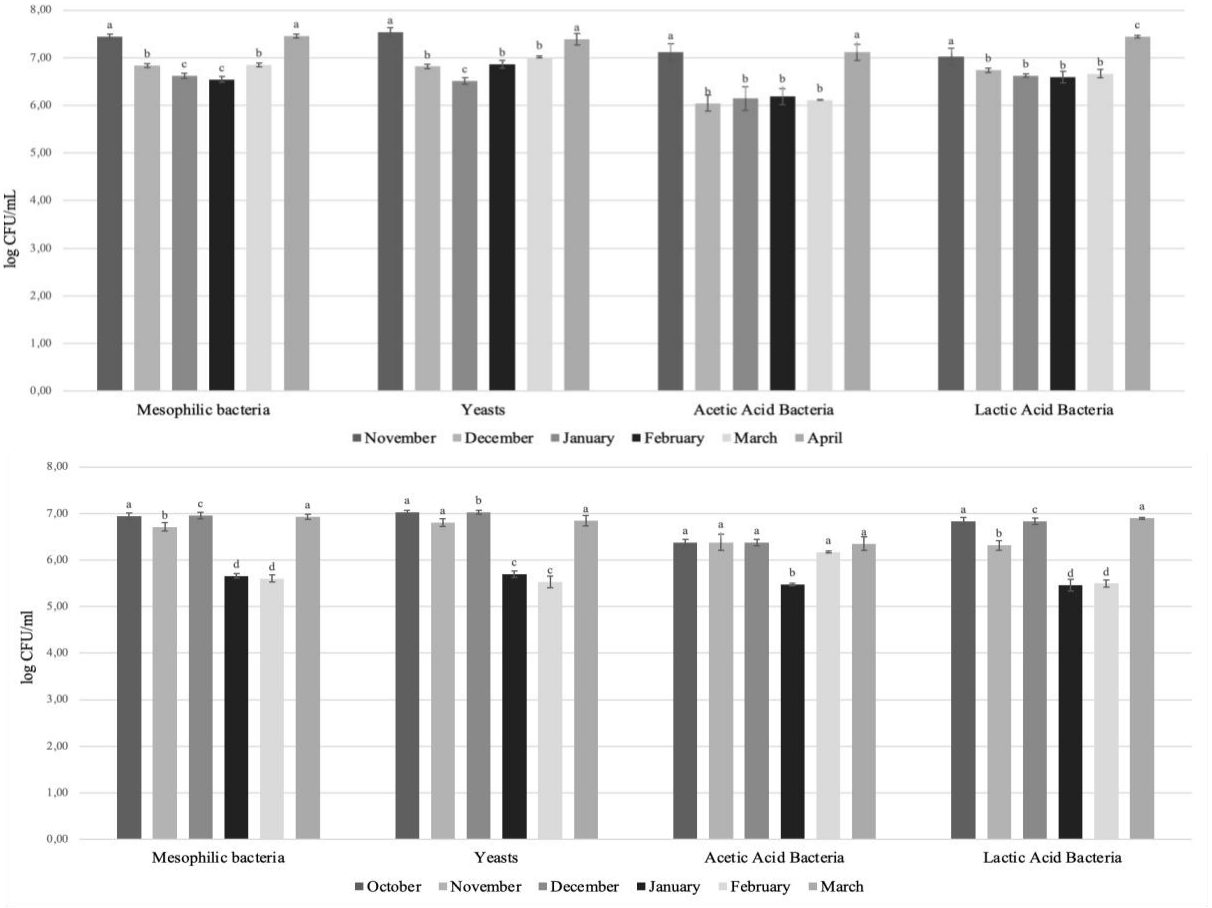

Figure 1 *Microbiological characterization of green (above) and black (below) kombucha. Results were expressed as mean of three repetitions. Error bars indicate ± standard deviation. Means followed by the same letter, for the same microbial group, are not significantly different ($p < 0.05$).*

Table 1 *Green and black kombucha pH after 5 and 7 days of fermentation, respectively.*

Kombucha	Month	pH	Kombucha	Month	pH
Green	November	3.31	Black	October	3.50
	December	3.00		November	3.15
	January	3.01		December	3.22
	February	3.00		January	3.03
	March	2.83		February	2.90
	April	3.05		March	3.20

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Analysis and characterization of Sicilian cereals landraces to destined at malting and brewing industry

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The first activities of the dissertation project involved the study of the malting aptitude of the old Sicilian landrace of Maiorca wheat and four varieties of two-rows barley that were grown in Sicily, these were respectively: Fortuna, Fandaga, Concerto and Planet. The wheat and barley varieties were malted in a pilot micro-malting plant using two malting programs. Several quality parameters were determined on malts such as: germination energy, thousand kernel weight, moisture, extract, total and soluble protein content, Kolbach Index, total starch, α- and β-amylase, diastatic power, fermentability and saccharification time.

Analisi e caratterizzazione delle varietà autoctone siciliane di grano e orzo da indirizzare all'industria del malto e della birra

Le prime attività del progetto di tesi di dottorato hanno riguardato lo studio dell'attitudine alla maltazione della varietà di grano antico siciliano Maiorca e di quattro varietà di orzo distico coltivate in Sicilia, questi ultimi erano rispettivamente: Fortuna, Fandaga, Concerto e Planet. Le varietà di frumento e orzo sono state maltate in un impianto di maltazione pilota utilizzando due programmi di maltazione. Nei malti ottenuti sono stati determinati diversi parametri qualitativi quali: energia germinativa, peso di mille semi, umidità, estratto, contenuto proteico totale e solubile, Indice di Kolbach, amido totale, α- e β-amilasi, potere diastatico, fermentabilità e tempo di saccarificazione.

Key words: malting process, wheat malt, barley malt, brewing process, cereals old landraces, Maiorca wheat malt.

1. Introduction

In accordance with the PhD thesis project, this poster reports the main results of the first two activities concerning:

(A1) Preliminary evaluation of un-malted wheat and barley landraces.
(A2) Pilot-scale malting trials performed in an automatic micro-malting system.
(A3) Physicochemical evaluation of malts and wort through the analysis of the most important quality index for malters and brewers as the content of extract, total starch, total protein content, soluble protein content, Kolbach Index, diastatic power, α-amylase and β-amylase content.

2. Materials and Methods

The malting trials were performed in triplicate in an automatic micro-malting system in SAAF Department of University of Palermo (Italy). The samples of wheat and barleys were cleaned to remove the glumes and husks, or, if present, external contaminants. Concerning Maiorca, the malting processes were carried out according to the conditions proposed by Alfeo et al. 2018a. For each basket, 500 g of grains were placed in steeped in water at 15 °C for 5 h, followed by 8 h of air-rest, and further 4 h in water, reaching steeping-out moisture of 41 %. The germination occurred after 120 h at 15 °C and 95 % of relative humidity, then the samples were dried and kilned for 34,5 h as follow: 3 h at 55 °C, 12 h at 60 °C, 10 h at 65 °C, 5 h at 70 °C, and 4,5 h at 75 °C. The barley samples were malted according to the following the malting conditions: 500 g of grains were placed in steeped in water at 15 °C for 10 h, followed by 12 h of air-rest, and further 6 h in water, reaching steeping-out moisture of 42 %. The germination occurred after 90 h at 15 °C and 95 % of relative humidity, then the samples were dried and kilned for 24 h as follow: 3 h at 55 °C, 9 h at 60 °C, 6 h at 65 °C, 2 h at 75 °C, and 4h at 85 °C. The analyses of malt and wort were performed in triplicate according to methods of Analytica European Brewery Convention (EBC). The Megazyme assay kits (Megazyme International, Ireland) were used for total starch (db%), α- and β- amylases (respectively T-STARCH following the AOAC Method 996.11 and K-MALTA).

3. Results and Discussion

The malt samples were analyzed, and the main parameters studied were summarized in Table 1. The choice of a cereal for malting considers the parameters that ensure the production of a high-quality malt, among them: thousand kernel weight (TKW), germination energy and protein content. TKW is a valuable parameter to maltsters and millers that is correlated with larger kernel size, homogeneous milling and proportionally higher endosperm (Armstrong et al., 2002). In the cereals studied, the TKW was between 31.5 g and 39.7 g; the lowest value was for

Maiorca wheat while among barley the lowest was for Concerto barley and the highest for Fortuna barley. Germination energy was evaluated to understand the health status of the seeds and their sensitivity to water (Briggs, 1998; Rani & Bhardwaj, 2021; Domin et al., 2019). The varieties studied showed high germinability values, all exceeding 97%. Regarding protein content, Maiorca malt showed a higher protein content than barley varieties, specifically 12.34 %db. The protein content in the barleys varied between 9.1 and 10.2%db. The protein content of the studied samples was generally in line with the values found in the literature recommended for wheat malt and barley malt (Faltermaier et al., 2014). Concerning malt quality parameters, Maiorca wheat malt showed values comparable to studies conducted previously by Alfeo et al, 2021 on Sicilian wheat landraces. The malting programs tested on wheat and barleys were suitable to ensure a good degree of grain modification and high malt quality that showed high values for: extract, fermentability, Kolbach Index, total starch, alpha and beta amylase and diastatic power. Extract on dry basis were for all samples above 82%, the highest value was for Maoirca malt in which it was around 84%db with a fermentability of 81.9%, thus showing a high amylolytic activity of amylase enzymes. Maiorca malt showed a diastatic power of 374.59 °WK positively correlated with β-amylase content which was 39.03 (BU g-1 db). The α-amylases that typically develop during malting starting from the second day was 200.95 (CU g-1 db). The barley samples studied also showed extract values above 82% with fermentability between 80 and 81.7%. The diastatic power of the barley malts was between 287 and 322 °WK, it showed an adequate enzyme content developed by the malting process. Concerning enzymes content, the content of α-amylase in barley malt samples was in range between 210 and 236 (CU g-1 db), respectively for Concerto and Fandaga. Compared to Maiorca malt, lower values were found for β-amylases. The β-amylase content in barley malts was between 13.55 and 15.75 (BU g-1 db) respectively for Fandaga and Planet. In all samples, the adequate grain modification was highlighted by the Kolbach Index, which had optimum values between 34.5% and 38.29% for Concerto barley variety and Maiorca malt, respectively. In conclusion, it was seen that all the varieties studied showed a good response to the malting parameters used; all varieties have the parameters evaluated by maltsters and brewers within the optimal ranges resulting good for brewing purpose.

Table 1 *Malts quality parameters.*

Parameter	Maiorca	Fortuna	Fandaga	Concerto	Planet
Thousand Corn Weight (g)	31.50±0.10	39.70±0.36	37±0.12	33.50±0.18	37±0.57
Germination energy (%)	97.50±0.01	98±0.08	97.5±0.04	97±0.09	97.50±0.04
Moinsture (% ww-1)	5.29±0.33	2.02±0.04	2.47±0.05	2.25±0.04	2.06±0.04
Extract (%db)	84.65±0.53	82.32±1.64	83.69±1.67	82.32±1.64	82.41±1.64
pH 20°C	6.13±0.04	5.98±0.07	5.78±0.11	5.38 ±0.10	5.78±0.23
Saccarification time (min)	<10	< 10	< 10	< 10	< 10
Colour (EBC unit)	4.87±0.11	3.70±0.18	3.78±0.26	3.27±0.08	5.69±0.10
Fermentability (%)	81.90±0.15	80.70±0.24	80.30±0.68	81.70±0.38	81.20±0.08
Proteins (%db)	12.34±0.44	9.10±0.17	9.2±0.18	10.2±0.20	10.10±0.19
Sol.Proteins (%db)	4.71±0.14	3.51±0.07	3.46±0.07	3.52±0.07	3.59±0.06
Kolbach Index (%)	38.29±2.54	38.12±0.70	37.39±0.70	34.50±0.69	35.52±0.69
Starch (%db)	62.60±0.88	56.82±2.05	52.62±1.05	53.27±1.45	55.38±1.08
α-amylase (BU g-1 db)	200.95±1.22	223.06±18.73	236.98±1.63	210.16±1.80	214.27±0.16
β-amylase (BU g-1 db)	39.03±0.08	14.91±4.05	13.55±0.12	13.76±0.92	15.75±0.73
Diastatic power (°WK)	375.59±2.53	290.25±4.19	322.09±5.70	287.13±4.63	304.13±4.48

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Human microbiota modulation by functional food consumption

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The main activities of PhD thesis project deal with the relationship between microbiota and functional food, in particular dietary supplements with probiotic and prebiotic addition. First of all, the relationship between synbiotic administration to nephropathic (Chronic Kidney Disease, CKD) subjects and variation of gut microbiota composition was evaluated. Secondly, the effects of probiotic yeast (*Saccharomyces bayanus* var. *uvarum*) assumption on composition and metabolism of microbiota in healthy subjects were defined. These effects were evaluated to consider their addition to food matrices and assess the effect of a functional food on the microbiota.

Modulazione del microbiota umano mediante l'assunzione di alimenti funzionali

Le attività principali del progetto di tesi di dottorato riguardano la relazione tra microbiota e alimenti funzionali, in particolare integratori alimentari con aggiunta di probiotici e prebiotici. In primo luogo, è stata valutata la relazione tra la somministrazione di un simbiotico a soggetti nefropatici (malattia renale cronica, MRC) e la variazione della composizione del microbiota intestinale. In secondo luogo, sono stati definiti gli effetti dell'assunzione di lievito probiotico (*Saccharomyces bayanus* var. *uvarum*) sulla composizione e sul metabolismo del microbiota. Questi sono stati valutati per considerare la loro aggiunta a matrici alimentari e valutare l'effetto di un alimento funzionale sul microbiota.

Key words: gut microbiota, functional food, synbiotic, health-promoting bacteria, probiotic yeasts.

1. Introduction

In accordance with the PhD thesis project previously described, this poster reports the main results of the first two activities concerning:
(A1) the restoration of the dysbiotic gut microbiota by synbiotic NatuREN G® administration in CKD subjects;
(A2) the variation of gut microbiota composition and metabolites by the consumption of probiotic yeast SERIUS.

2. Materials and Methods

2.1 Restoration of gut microbiota by synbiotic NatuREN G® in CKD subjects

A placebo-controlled, randomized, single-blind study in healthy and CKD subjects was planned. Patients were randomized to receive NatuREN G® (*Bifidobacterium animalis* BLC1, *Lacticaseibacillus casei* LC4P1, fructo-oligosaccharides, inulin, quercetin, resveratrol, and proanthocyanidins) or placebo. The two groups were named, respectively, CDK-S and CDK-P. Faecal samples were collected at the beginning of the study (T0), after two months of treatment (T60) and after one month of wash-out (T90). DNA extraction from feces and quantitative PCR were performed to evaluate an increment in *Lactobacillus* and *Bifidobacterium* genera.

2.2 Evaluation of probiotic potential of *Saccharomyces bayanus* var. *uvarum*

The yeast probiotic properties were evaluated by a placebo-controlled, randomized cross-over study in 50 healthy subject who received SERIUS (*Saccharomyces bayanus* var. *uvarum* IRIS-SERIUS) or placebo (similar package containing xanthan gum). Faecal samples were collected at the beginning of the study (T0), at the end of first treatment (T60), at the end of wash-out period (T75) and at the end of second treatment (T135). DNA extraction, quantitative PCR and metabolome analysis (volatile organic compound, VOC) were performed to evaluate variation of composition of gut microbiota and variation of metabolites.

3. Results and Discussion

3.1 Restoration of gut microbiota by synbiotic NatuREN G® in CKD subjects

At the beginning of the study (T0), no significant changes were found among all subjects and bacterial targets. After 60 days of treatment (T60) and after 30 days wash-out (T90), there was no significant increase in the CKD-S group, while in CKD-P there was a decrease of *Lactobacillus* genus. Otherwise, for *Bifidobacterium* genus, there was a significant decrease in its abundance in the CKD-P group at the end of the trial (T90) compared with the beginning of the study and after 60 days of placebo treatment (T0 and T60). In the CKD-S group, *Bifidobacterium* abundance increased in every check point of the study. However, 60 days of treatment with NatuREN G® was not sufficient to reach significance, the values collected at T90 were significantly

different from those assessed at T0. The symbiotic NatuREN G® exerted selective efficacy in patients with stage IIIb-IV CKD. It was able to modulate the gut microbiota profile and related metabolism by increasing the ratio of Firmicutes to Bacteroidetes. A decrease of this ratio was previously noticed as a signature of chronic relapsing inflammation affecting the intestinal mucosa (Carding 2015). Although species belonging to Firmicutes and Actinobacteria contain relatively few fiber-metabolizing enzymes *per* organism, these phyla are the main responders to plant-derived nutrients (Deehan 2017). Both Firmicutes and Actinobacteria generally exert specialized roles, such as the initiation of complex substrate degradation (Martínez 2010). Therefore, the increased intake of fiber assessed by the dietary recall after the treatment with NatuREN G® may have sustained the abundance of Firmicutes in this pilot study. Therefore, the present work paves the way for further studies based on nutrition and adjuvant therapies based on the administration of probiotics and prebiotics, while having as interest the treatment of diseases without gastrointestinal background, such as nephropathy.

3.2 Evaluation of probiotic potential of *Saccharomyces bayanus* var. *uvarum*

Faecal volatile organic compounds (VOCs) from healthy subjects following treatment with the probiotic (S. bayanus SERIUS/IRIS) or placebo (maltodextrins/xanthan gum/erythrol) were analysed. Untargeted analysis of faecal metabolites (GC-MS) identified a total of 134 VOCs, grouped into the following chemical classes: alcohols (16), aldehydes (10), esters and methyl esters (24), hydrocarbons (7), indoles (3), ketones (10), organic acids (18), phenols (4), sulfuric compounds (4), terpenes (24), and others (14). An initial analysis comparing the percentage of each of the chemical classes examined was conducted by comparing them in the two treatment groups at the beginning and end of administration. Phenols, indoles and organic acids were the most represented classes in all groups examined. The highest percentage of organic acids (29.73%) was presented in samples from healthy subjects after treatment with probiotic (*S. bayanus* SERIUS/IRIS). The compounds that increased in the probiotic-treated group (3-methyl-Indole; 2-Pentadecanone; 2-Undecanol; 2,6-Dimethylphenyl isocyanate and others).

3-Methylindole is the product of intestinal metabolism of tryptophan. Gut bacterial species convert tryptophan to tryptamine and indole-3-pyruvic acid, as well as convert it to indole, indole-3-acetaldehyde and indole-lactic acid. Some species belonging to the phylum Firmicutes (*Lactobacillus johnsonii*, *Limosilactobacillus reuteri*, *Ligilactobacillus murinus*, and *Lactobacillus acidophillus*) convert indole-3-acetaldehyde to indole-3-acetic acid, which by decarboxylation generates 3-methylindole. Intestinal tryptophan metabolites participated in the host-gut microbiota cross-talk and acted as aryl hydrocarbon receptor (AhR) ligands and agonists (Agus 2018; Dong & Perdew 2020; Hubbard 2015; Lamas 2018; Nicholson 2012). The AhR played a key role in regulating the immune system, as well as regulating the production of enzymes involved in metabolic processes. In particular, AhR can stimulate the immune response at the level of the intestinal mucosal barrier by modulating intraepithelial lymphocytes, T cells, and the production of interleukin-17 (IL-17) and IL-22, also contribute the maintenance of gut eubiosis (Lamas 2020). Finally, a targeted analysis of only short-chain fatty acids (SCFAs) (acetic, propionic, isobutyric, butanoic, and isovaleric) showed a statistically significant increase in acetic acid in healthy subjects following probiotic treatment. Short-chain fatty acids are final products of gut fermentation with trophic effect on the intestinal epithelium. In addition, they are involved in regulating the immune response and improving the barrier function of the intestine (LeBlanc 2017; Parada Venegas 2019; Ríos-Covián 2016). The statistically highlighted significant increase in acetic acid in faecal samples following treatment with probiotic (S. bayanus SERIUS/IRIS). This suggests an effect of counteracting gut dysbiosis and stimulating immune response.

Preliminary qPCR results showed an increase in the genus *Bifidobacterium*, *Bifidobacterium adolescentis*, and *Limosilactobacillus fermentum* after placebo intake, and an increase in *Bifidobacterium bifidum* after probiotic intake, as well as an increase in *Saccharomyces bayanus*. These evidences have been reported in Log (Copy Number). A standard curve for each primer was constructed with sequential dilutions of DNA extracted from type strain culture. The Copy Number (CN) and the Logarithms were calculated based on DNA concentration and amplicon length. The standard curve was constructed by interpolating the Log (Copy Number) and Cycle threshold (C_T) obtained from qPCR analysis.

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Use of Wild Edible Plants as Environmental Indicators and as Ingredients for the Creation of new Functional and Enriched Products

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The main research activities planned in the first two years of the PhD project have been completed. Firstly, two wild edible plants (WEPs) species (*M. sylvestris, F. vulgare*) and different sampling places were identified. Amounts of major bioactive compounds (tocols, carotenes, xanthophylls, riboflavin and thiamine) were determinedand two suitable analytical procedures were used for the determination of polycyclic aromatic hydrocarbons (PAHs) and heavy metals. In addition, preliminary analyses were conducted to assess the contribution of anthropogenic sources on the contamination levels of the areas chosen for sampling.

Utilizzo delle piante selvatiche commestibili come indicatori ambientali e come ingredienti per la preparazione di nuovi prodotti funzionali e arricchiti

Le principali attività di ricerca previste nei primi due anni di dottorato sono state completate. In primo luogo, sono state identificate due specie di piante selvatiche commestibili (*M. sylvestris*e *F. vulgare*) e diverse località per il campionamento. Sono state determinate le quantità presenti dei principali composti bioattivi (tocoli, caroteni, xantofille, riboflavina e tiamina) e, parallelamente, sono state utilizzate due procedure analitiche idonee per la determinazione degli idrocarburi policiclici aromatici (IPA) e dei metalli pesanti. Inoltre, sono state condotte analisi preliminari per valutare il contributo delle sorgenti antropiche sui livelli di contaminazione delle aree scelte per il campionamento.

Key words: Wild edible plants, bioactive compounds, vitamins, environmental pollution, PAHs, health.

1. Introduction

In accordance with the PhD thesis project previously described (Ianiri G, 2022), this poster reports the results of the main research activities planned in the first two years of the PhD project concerning:

(A4) the set up of analytical procedures suitable for the analysis of polycyclic aromatic hydrocarbons and heavy metals in WEPs under study;

(A5) the sampling activities, which were carried out choosing three places with different anthropic impact, specifically Rotello, Termoli and Rome;

(A6) the realization of preliminary analyses for the quantitative determination of bioactive compounds and of PAHs and heavy metals.

2. Materials and Methods

As it regards the determination of tocols, carotenes, xanthophylls, riboflavin and thiamin, methods already developed by (Panfili G, 2003, 2004)were used. The materials and methods used for the determination of polycyclic aromatic hydrocarbons and heavy metals are briefly described. The analytical protocol for the determination of PAHs was divided into three steps. In the first step, 2 grams of fresh WEPs leaves were placed in a beaker to which 30 mL of high-purity cyclohexane was added to minimize interference. Extraction was performed in an ultrasonic bath for 30 min. Finally, the extract was filtered on filter paper and collected in an Erlenmeyer flask to avoid exposure to light. In the second step, the extract was concentrated to a volume of about 5 mL through a rotary evaporator. The concentrate was then passed over a column containing anhydrous sodium sulfate. Finally, the extract was reduced to a volume of 100 µL using a gentle stream of purified nitrogen. The last step involves analysing the extract by injecting 1µL into the single quadrupole gas chromatography mass spectrometry (6890-GC/5973-MSD) system. For the determination of heavy metals, mineralization of organic matter was carried out for each sample. To 2 grams of freeze-dried sample, 10-15 mL of a mixture of nitric acid and sulphuric acid 1:3 v/v was added and then samples were placed on a hot plate for 1 hr. The whole samples were then brought to a final volume of 50 mL and analysed by inductively coupled plasma atomic emission spectroscopy (ICP-AES).

3. Results and Discussion

Table 1 shows the content of bioactive compounds in *M. sylvestris* and *F. vulgare* sampled in Rotello and Termoli.Detected carotenoids included lutein, zeaxanthin, violaxanthin, neoxanthin, β-cryptoxanthin, anteraxanthin, α-carotene, 13-cis- β-carotene, β-carotene and 9-cis-β-carotene. Detected tocols were α-Tocopherol,

β-Tocopherol, γ-Tocopherol.

Table 1 *Contents of total carotenoids and total tocols (mg/100g of fresh weight) with respective standard deviations (SD) (n=3). Contents of riboflavin and thiamine (mg/kg of fresh weight)with respective SD (n=3).*

	M. sylvestris **Rotello**	***M. sylvestris*** **Termoli**	***F. vulgare*** **Rotello**	***F. vulgare*** **Termoli**
Total Carotenoids	31.70 ± 4.20	43.00 ± 2.20	27.30 ± 2.20	13.30 ± 0.30
Total Tocols	3.90 ± 0.50	9.50 ± 2.50	3.70 ± 1.80	2.20 ± 0.01
Riboflavin	0.46 ± 0.01	0.85 ± 0.23	0.90 ± 0.03	0.38 ± 0.02
Tiamin	62.47± 27.12	56.73 ± 0.51	16.01± 6.73	7.25 ± 0.01

From the data shown in Table 1, the amounts of vitamin A, as Retinol Equivalent (RE in µg/100g) and vitamin E, as Tocopherol Equivalent (TE in mg/100g) were calculated. Given the Recommended Daily Allowance (RDA) reported in Annex XIII, part A, of EU Regulation 1169/2011, the percentages of the RDA provided by the consumption of 100 grams of *M. sylvestris* and *F. vulgare* were calculated (Table 2).

Table 2 *Percentage of the RDA covered by the intake of 100 grams of fresh M. sylvestris and F. vulgare.*

	M.sylvestris **Rot.**	***M.sylvestris*** **Ter.**	***F. vulgare*** **Rot.**	***F. vulgare*** **Ter.**
Vitamin A	144	279	147	93
VitaminE	31	74	28	17
Riboflavin	3	6	7	3
Tiamin	568	516	145	66

The consumption of 100 grams of *M. sylvestris* and *F. vulgare* sampled in Rotello and Termoli largely covered the RDA of vitamin A, vitamin E, and thiamine. On the other hand, for riboflavin, the amounts of all samples did not reach the recommended daily dose. Given the high amounts of bioactive compounds of the two analysed WEPs, new food formulations will be developed to realise products intended to fulfil nutritional deficiencies and, in general, to provide health benefits for consumers. Given the spontaneous nature of WEPs, they can contain multiple organic and inorganic micro-pollutants that are extremely harmful to health (Terzi M, 2013). Before using WEPs in new food formulations, it is, therefore, necessary to evaluate the levels of the main contaminants potentially present, such as PAHs and heavy metals. This activity can also be used to establish the influence of anthropogenic sources on the levels and profiles of PAH and metal contamination in wild plants. This consequently could allow the possibility of using wild plants as environmental indicators. Table 3 shows the levels of the determined heavy metals and the sum of the polycyclic aromatic hydrocarbons of *M. sylvestris* sampled in Rotello and Rome. Among the PAHs determined is benzo[a]pyrene, the target molecule used for carcinogenic risk assessments. The Low Detection Limits (LODs) of Pb, Cd and Co were respectively 0.2, 0.005 and 0.1 µg/kg.

Table 3 *Heavy metals and PAHs concentrations ± SD in µg/Kg of fresh weight in M. Sylvestris leaves.*

	Pb	**Cd**	**Cr**	**Co**	**Cu**	**Total PAHs**
M.sylvestris **Rome**	1,94 ± 0,02	0,01 ± 0,00	0,73 ± 0,02	0,55 ± 0,07	21,34 ± 1,36	550 ± 1
M.sylvestris **Rotello**	<LOD	<LOD	0,25 ± 0,09	<LOD	1,69 ± 0,09	297 ± 2

Rotello and Rome were chosen to highlight the contribution that a large urban center, like the city of Rome, has on the state of contamination of wild plants. In fact, this is marked both for the metal component and for the PAHs. The aim is to make the population aware of the risk of collecting wild plants that grow, or are located, close to emission sources, or in territorial contexts characterized by the presence of numerous anthropic activities.

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Olive Vinegar as Functional Ingredient and Antioxidant Agent in Vegetable Sauce

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This study explores the potential of olive leaf vinegar (OLV) in vegetable sauce (VS). OLV-VS exhibited a total phenolic and oleuropein content of 0.68 mg/g GAE and 0.18 mg/g Hydroxytyrosol equivalent respectively confirming the functional potential of VS. The strong antioxidant activity of OLV was reflected by incredibly low value of DPPH IC50 (0.10 mg/g). The minimal changes in peroxide value (PV) were observed during the oven test at 40 °C up to 60 days, which also signifies the role of OLV as strong antioxidant agent in sauce. Ultimately, functional and antioxidant role of OLV was proven in the VS formulation.

Aceto di oliva nella salsa di vegetali in qualità di Ingrediente Functionale e di Attivo Antiossidante

Il presente studio esplora il potenziale dell'aceto di foglie di olivo (OLV) nella salsa di vegetali (VS). L'OLV-VS ha mostrato un contenuto di fenoli totali e di oleuropeina rispettivamente di 0,68 mg/g di GAE e 0,18 mg/g di Idrossitirosolo equivalente, confermando il potenziale funzionale della VS. La forte attività antiossidante dell'OLV è stata riflessa dal valore molto basso dell'IC50 del DPPH (0,10 mg/g). Le variazioni minime del valore di perossido (PV) sono state osservate durante il test in forno a 40 °C fino a 60 giorni, il che indica anche il ruolo dell'OLV come forte agente antiossidante nella salsa. Infine, è stato dimostrato il ruolo funzionale e antiossidante dell'OLV nella formulazione VS.

Key words: olive leaf vinegar, vegetable sauce, functional, oleuropein, antioxidant DPPH test, oven test.

1. Introduction

Food industries are paying great attention to producing healthier products endowed with high nutritional value and therapeutic benefits. Several studies have been conducted with the specific aim of replacing some conventional ingredients in the preparation of vegetable sauce.
In a previous study De Leonardis et al (2022a), olive leaf vinegar (OLV) was produced, and its functional potential was assayed in an oil/vinegar dressing-based formulation. In addition, the feasibility of the use of OLV in vegetable sauce (VS) was investigated in current research. Chemical characterization of the VS was performed through moisture, fat, pH, total phenols, and peroxide value (PV) determination. Finally, the oxidative stability of VS was evaluated by PV evolution during an accelerated storage test (oven test). Study unveils an intriguing exploration into the exceptional properties of OLV within culinary applications.

2. Materials and Methods

OLV was prepared by maceration of dried olive leaves in 18% acetic acid vinegar (AV) following the procedure described in De Leonardis et al (2022a). Sauce preparation was conducted according to the method described in De Leonardis et al (2022b) with slight modification. By unlocking the potential of following ingredients in definite percentages: 56% soybean and sunflower seed oil blend (15:85, v/v), 35% commercial soymilk, 8% OLV as test ingredient while AV as control ingredient and 1% salt. Final packaging of VS was performed manually in aluminum sachets of 15 g capacity.
Physicochemical parameters, such as moisture, fat, pH, peroxide value, phenols, and antioxidant potential were determined as described by De Leonardis et al (2022a). Specifically, Total Phenols (TP) was determined by Folin–Ciocalteu spectrophotometric method expressing the results in gallic acid equivalent (GAE), while the oleuropein (OLE) was quantified by HPLC analysis in hydroxytyrosol equivalent (HYE). Antioxidant potential was evaluated by calculating IC50 dose (g of sauce) of DPPH inhibition percentage (I%) calculated with the following equation:

$$\%I = ((\text{Abs blank} - \text{Abs sample})/\text{Abs blank}) \times 100 \quad (1)$$

Finally, oven test was conducted placing the sauce sachets in a thermostat at 40 °C for 2 months.

3. Results and Discussion

3.1 Physicochemical properties of formulated vegetable sauce

The physicochemical characteristics of the vegetable sauce play a pivotal role in determining its quality, flavor, and stability, thereby ensuring an optimal taste experience and extended shelf-life. As indicated in Table 1, no

statistically significant differences were observed between the samples (AV-VS and OLV-VS) in terms of pH, moisture and fat content. The remarkably low average pH value of 2.15 in both sauces serves as a robust safeguard against microbial spoilage. While AV-VS exhibited only trace amounts of total phenols, OLV-VS showcased a notable concentration of 0.68 mg/g GAE, with the presence of oleuropein as a predominant phenolic compound, supported by the HPLC chromatogram (Figure 1). These findings highlight the distinctive phenolic profile and potential health benefits associated with OLV-VS.

Table 1 *Analytical determinations of the vegetable sauce samples. (AV-VS: 18% acetic acid vinegar sauce; OLV-VS: olive leaf vinegar sauce sample)*

Sample	Moisture %	Fat %	pH	Peroxide value ($meqO_2/kg$)	Total phenols (mg/g GAE)	Oleuropein (mg/g) HYE
AV-VS	31.6 ± 1.9^a	58.2 ± 2.6^a	2.16 ± 0.45^a	2.0 ± 0.1^a	Traces	Traces
OLV-VS	31.0 ± 1.7^a	57.0 ± 2.4^a	2.15 ± 0.38^a	1.9 ± 0.1^a	0.68 ± 0.08^b	0.18 ± 0.02^b

3.2 Functional potential

In the domain of olive tree products, hydroxytyrosol and its derivatives (HY&Der), including oleuropein, are prominent bioactive compounds known for their well-established antioxidant, antimicrobial, anti-inflammatory, and anticancer properties, as well as their potential in the prevention of cardiovascular diseases, metabolic syndromes, and neurodegenerative disorders Hadrich et al (2022). Notably, the European Food Safety Authority (EFSA) permits the inclusion of a health claim on the labels of extra virgin olive oils, stating that the polyphenols naturally present in these oils contribute to the protection of blood lipids against oxidative stress. To attain this benefit, a daily intake of at least 5 mg of HY&Der is recommended. The functional role of OLV in the formulated VS is demonstrated in Figure 2, wherein a 15 g portion of OLV-VS (one sachet) contained 2.5 mg HY&Der, approximately 50% of the recommended daily dosage. Furthermore, the DPPH IC50 value determined in OLV-VS was 0.10 mg/g, affirming the functional potential of the formulated vegetable sauce.

Figure 1 *Chromatogram from HPLC for Oleuropein* content in OLV-VS at 240 nm

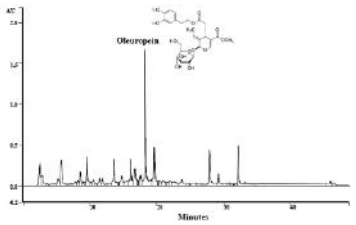
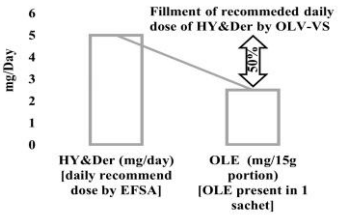


Figure 2 *Relation between recommended daily dose of HY&Der and provided by OLV-VS*

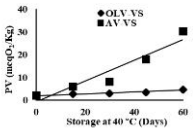


3.3 Antioxidant effect on the vegetable sauce shelf life

Measurement of the PV can help to estimate the oxidation stage. The oven test represents an accelerated method frequently used to test autoxidation in oils and fats under medium high temperatures. Results of the oven test conducted in this research are shown in Figure 5.

OLV-VS showed oxidation stability higher than the control. Specifically, extraordinarily slight change in PV was observed for OLV-VS (from 1.9 to 4.7 $meqO_2/kg$) in 60 days, against the PV increase (from 2.0 to 30.1 $meqO_2/Kg$), observed in VS-AV (control).Therefore, OLV, thanks to its phenolic component, has proven to be a good antioxidant agent for the sauce.

Figure 3 *Variation of Peroxide value (PV) measured at 40 °C for 2 months in sauce samples prepared with olive leaf vinegar (OLV-VS) and alcoholic vinegar (AV-VS).*



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Plant health monitoring of durum wheat, pathogen identification with advanced diagnostic tools and design of sustainable prevention system

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According to the activities described in the PhD thesis project, some changes were made in order to focus better on main objectives and reach best possible results. We decided to focus on only one crop species, *Triticum durum* in order to be able analyse in detail the microbiome community of fungal species of the crop in the period of three years. Firstly, the caryopsis analysis were done by using morphological identification of fungal species and molecular analysis followed by assessment of mycotoxin analyses. Secondly, the soil microbiome community was studied for a period of three years 2020-2022 in order to understand the differences between plots treated with organic compost and mineral fertilizer.

Monitoraggio fitosanitario del grano duro, identificazione di agenti patogeni con strumenti diagnostici avanzati e progettazione di un sistema di prevenzione sostenibile

Secondo le attività descritte nel progetto di tesi di dottorato, sono state apportate alcune modifiche al fine di concentrarsi meglio sugli obiettivi principali e raggiungere i migliori risultati possibili. Abbiamo deciso di concentrarci su una sola specie vegetale, il *Triticum durum* per poter analizzare in dettaglio la comunità microbiotica delle specie fungine della coltura nel periodo di tre anni. Innanzitutto, l'analisi della cariosside che è stata effettuata utilizzando l'identificazione morfologica delle specie fungine e l'analisi molecolare seguite da verifica delle micotossine presenti. Inoltre, la comunità del microbiotica del suolo è stata studiata per un periodo di tre anni 2020-2022 per capire le differenze fra le parcelle trattate con il compost organico e fertilizzante minerale.

Key words: wheat, *Triticum durum*, fungal species, microbiome community, organic compost, mineral fertilizer.

1. Introduction

In accordance with the PhD thesis project and the changes previously described, this poster reports the main results of the first two activities concerning:

(A1) Biodiversity and metagenomics. The sampling of wheat (*Triticum durum*), variety Antalis in year 2022 and microbiome analysis of the wheat rhizosphere for years 2020, 2021 and 2022 by using PCR and HTS analysis.

(A2) Food safety. Fungal contaminants of caryopses and mycotoxins. Morphological and molecular analysis of fungal species isolated from wheat caryopsis collected through the period of three years (2020 – 2022) and assessment of mycotoxins present.

2. Materials and Methods

The sampling of wheat took place three times in each experimentation year in order to understand if there is a difference between different phenological phases: A) Tilling; B) Rising; C) Ripening. Besides that, different soil treatments were considered: organic compost or mineral fertilizer, as well as different soil tillage practices: A) Ploughing; B) Digging; C) Ripping. Total DNA was extracted from soil samples using the Nucleo Spin Soil Kit (Macherey - Nagel), following the protocol. The PCR reaction was done using the ITS1 region was amplified with a dual indexing primer using the tagged primer pair ITS1F and ITS2. The thermal cycle was an initial denaturation at 94 ºC for 10 min followed by 30 cycles of 95 ºC for 40 s, 60 ºC for 40 s and 72 ºC for 1 min, and a final elongation step of 72 ºC for 10 min. Amplicons were purified using the Mag JET NGS Clean-up (Thermo Scientific, USA), quantified with the Qubit Quantitation kit (Invitrogen, USA), and pooled at equal concentrations for sequencing. Paired-end sequencing (2 x 300 bp) was carried out on an Illumina Mi Seq sequencer by Fasteris SA (Switzerland) for samples collected in 2020. The analyses of caryopses were done by isolation of fungal species and their morphological and molecular analyses. The morphological analyses were done by placing the caryopses in Petri plates using the DFB method as described by (Liomonard 1996) and the culture medium PDA (Potato Dextrose Agar). Once the pure cultures were obtained the morphotypes were assigned based on macro morphology. Afterwards, the molecular analyses were done in order to reach the fungal species, started from the DNA extraction of morphotypes, PCR, purification of amplicons, quantification and sequencing (Senatore *et al*., 2023; Beccari *et al*., 2020). The PCR reaction was done using ITS1 and ITS4 primers and elongation factor.

3. Results and Discussion

3.1 Biodiversity and metagenomics

Bioinformatics analyses are still in progress. Results for the fungal population in are completed for year 2020 in terms of order distribution. We are witnessing the first year of experimentation with a strong increase in fungal orders in plots fertilized with compost with a significant change in the percentage of orders. The order with greater frequency is the Hypocreales, represented by Fusarium species (*Fusarium brachygibbosum, Fusarium equiseti, Fusarium oxysporum species complex, Fusarium redolens, Fusarium poae, Fusarium solani species complex)* which results to be more abundant in plots treated with mineral fertilizer (***Fig. 1***). From what emerges from the Venn diagram (***Fig. 2***), the rhizosphere soil of the plots fertilized with compost together present 60% more of the fungal orders. Ongoing analyses will provide more detail on the functionality of fungal species over the three years and the role of compost in providing ecosystem systems for the biological part of soil fertility.

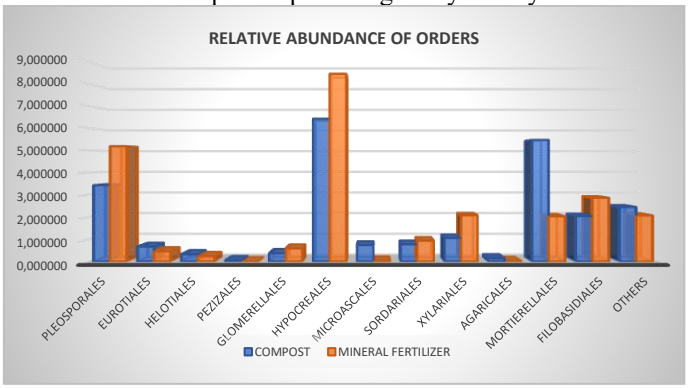

Figure 1 *Relative abundance of orders according to different treatments*

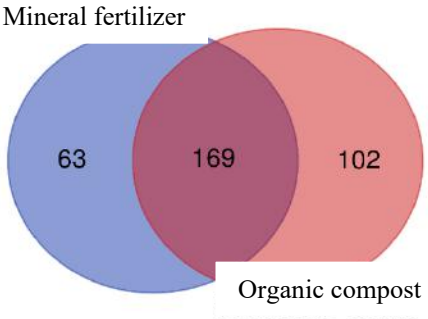

Figure 2 *Venn diagram*

3.2 Food safety: fungal contaminants of caryopses and mycotoxins

The results obtained indicate that in the three years of experimentation there has been a variation in the abundance of the various fungal species colonizing the caryopsis. These variations were mainly due to the meteorological trend. All three years were characterized by a drought during the spring summer period, with 2022 demonstrated with arid climate. The general trend was characterized by a greater colonization by s of the genus *Alternaria*. This genus is characterized by a greater development in warm periods during the ripening phase, than in the years of experimentation. The 2022 was the driest and this certainly favoured *Alternaria* species. Conversely, the species belonging to the genus *Fusarium* have recorded a poor development that can be related to the low spring rainfall during the flowering phase, site of penetration and colonization of these mycotoxin fungi. Some species of *Fusarium*, such as *F. equiseti* (2020 and 2021) and *F. poeae* (2020) have been absent in chemically fertilized theses, compared to the organic, which however has recorded extremely low values. On the whole, it can be said that the experimental theses have shown little influence on the rate of colonization of the different mycotoxin fungi, while the climatic factor is predominant.

Table 1 *Relative abundance (%) of mycotoxin fungal species detected in caryopses by blot - deep freezing method. Data refer to standard error averages.*

	AM			AO			RM			RO			VM			VO		
2020	mean		Std. Error	mean		Std. Error	mean		Std. Error	mean		Std. Error	mean		Std. Error	mean		Std. Error
Alternaria alternata	7,33	±	3,22	19,33	±	5,32	0,00	±	0,00	24,00	±	2,70	0,00	±	0,00	24,00	±	3,45
Alternaria infectoria	5,33	±	1,50	3,33	±	1,62	0,00	±	0,00	20,33	±	3,17	14,00	±	3,63	17,33	±	2,43
Alternaria tenuissima	5,33	±	1,24	3,00	±	1,98	4,00	±	1,21	11,00	±	1,64	0,00	±	0,00	9,00	±	1,00
Fusarium proliferatum	4,67	±	2,14	0,00	±	0,00	3,00	±	1,40	0,00	±	0,00	0,33	±	0,33	0,00	±	0,00
Fusarium equiseti	0,00	±	0,00	0,00	±	0,00	0,00	±	0,00	2,67	±	1,42	0,00	±	0,00	2,67	±	1,24
Fusarium poeae	0,00	±	0,00	0,00	±	0,00	0,00	±	0,00	3,67	±	1,67	2,33	±	1,15	3,67	±	1,87
no contaminated	77,34			74,33			93,00			38,33			83,33			43,33		
2021																		
Alternaria alternata	3,33	±	1,76	3,33	±	1,76	1,33	±	0,90	6,00	±	2,23	1,33	±	1,33	3,00	±	1,64
Fusarium avenaceum	0,00	±	0,00	0,00	±	0,00	1,00	±	0,52	3,33	±	1,76	5,67	±	1,94	2,33	±	1,43
Fusarium equiseti	0,00	±	0,00	0,00	±	0,00	0,33	±	0,33	1,33	±	1,33	0,67	±	0,67	0,00	±	0,00
Fusarium fujikuroi	2,33	±	1,59	1,00	±	1,00	1,00	±	1,00	1,33	±	1,33	3,00	±	1,57	1,67	±	1,15
Fusarium poeae	1,67	±	1,04	1,33	±	1,33	1,00	±	1,00	0,00	±	0,00	4,33	±	1,94	1,33	±	1,33
Fusarium tricictum	0,00	±	0,00	0,00	±	0,00	2,33	±	1,59	1,33	±	1,33	3,00	±	1,49	1,67	±	1,34
no contaminated	92,67			94,33			93,00			86,67			82,00			90,00		
2022																		
Alternaria alternata	24,67	±	2,95	12,67	±	4,92	1,33	±	0,75	16,33	±	4,13	28,67	±	4,17	7,00	±	3,12
Alternaria infectoria	9,33	±	4,13	18,67	±	2,80	11,33	±	3,93	18,33	±	2,53	12,00	±	2,74	19,67	±	3,43
Alternaria malorum	0,00	±	0,00	5,67	±	2,28	7,00	±	2,26	8,33	±	2,85	0,00	±	0,00	2,33	±	1,15
Alternaria tenuissima	5,00	±	2,61	0,00	±	0,00	11,67	±	3,01	0,00	±	0,00	4,00	±	2,15	0,00	±	0,00
Fusarium proliferatum	2,67	±	1,42	1,00	±	0,72	2,67	±	1,14	0,67	±	0,67	1,67	±	0,77	1,67	±	0,77
Fusarium equiseti	0,33	±	0,33	1,67	±	0,92	0,67	±	0,45	2,00	±	0,92	1,67	±	1,04	1,67	±	1,15
Fusarium sporotrichoides	2,00	±	1,04	0,33	±	0,33	3,00	±	1,22	2,00	±	0,92	2,67	±	1,14	2,67	±	1,14
no contaminated	56,00			60,00			62,33			52,34			49,33			65,00		

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Elicitor-mediated enhanced accumulation of secondary metabolites in apple cell cultures

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Our work first aimed to the development of pulp-derived callus cultures from an apple landrace native to southern Italy. Thereafter, the content of secondary metabolites (SMs) within pulp-derived calli was analysed, showing the presence of a good amount of bioactive compounds. In light of this, the use of elicitors was tested in order to boost the production of these molecules.

Incrementato accumulo di metaboliti secondari in colture cellulari di mela mediato da elicitori

Il nostro lavoro ha avuto come primo obiettivo lo sviluppo di colture di callo vegetale derivate dalla polpa di una cultivar di mela originaria dell'Italia meridionale. Successivamente, è stato analizzato il contenuto di metaboliti secondari all'interno dei calli derivati dalla polpa, evidenziando la presenza di una buona quantità di composti bioattivi. Alla luce di ciò, è stato testato l'uso di elicitori per incrementare la produzione di queste molecole.

Key words: apple, callus cultures, secondary metabolism, elicitor, polyphenols, triterpenic acids.

1. Introduction

In accordance with the PhD thesis project previously described (Laezza, 2022), this poster reports the main results of the first two activities concerning:
(A1) the production of apple pulp calli and the analysis of the content of bioactive compounds within these cell cultures.
(A2) the use of salicylic acid as elicitor to determine an increase in the production of secondary metabolites. The analysis of the content of bioactive compounds after elicitation.

2. Materials and Methods

Apple pulp was sterilized, cut into small pieces, and placed on Gamborg B5 medium (Dixon, 1985) with auxin and cytokinin in a ratio 2:1. Cell cultures were incubated in the dark at 25 ± 2 °C. Pulp-derived calli from exponential developmental stage were inoculated on control medium and on medium with the addition of 50 and 100 mg/L salicylic acid (SA). Thereafter, extracts from freeze-dried apple calli were obtained with 1 mL of 1% formic acid methanol-water (80:20; v/v) mixture and with the use of vortex, ultrasonic bath and centrifuge at 9000 rpm for 10 minutes. The HPLC-DAD/ESI-MS^n analysis was performed as described by Tenore et al. (2013).

3. Results and Discussion

3.1 Development of apple pulp-derived callus cultures and analysis of secondary metabolites.

Several studies have suggested that plant cell cultures (PCCs) enhance the production of secondary metabolites. Consequently, PCCs have been long studied especially in terms of their nutritional properties as food products. Here, we reported our results demonstrating the development of apple pulp callus culture, starting from raw material obtained from *Malus pumila* Miller cv Annurca, which is a native apple landrace to southern Italy (Figure 1.).

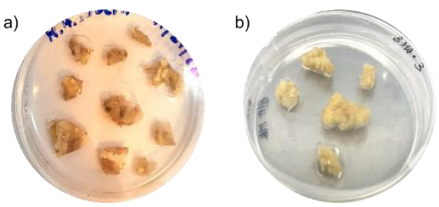

Figure 1 *(a) Apple pulp pieces placed in agar medium; (b) Pulp-derived calli.*

Table 1 *The content of metabolites presents within pulp-derived calli compared to lyophilized apple.*

Secondary metabolites(mg) / callus(g)		
Compounds	**Pulp**	**Callus**
Procyanidin B1	0.421±0.060	0.318±0.044
Procyanidin B2	0.519±0.013	0.028±0.0006***
Epicatechin	0.408±0.019	0.001±0.0003***
Gallic acid	0.120±0.017	0.102±0.025
Ursolic acid	4.020±0.010	2.190±0.292**

Values are means ± SE (n = 3) and asterisks denote statistically significant differences of each treatment compared to the apple lyophilized (**p ≤ 0.001; ***p ≤ 0.0001) according to Student's *t*-test.

Furthermore, the SMs content was assessed. As shown in Table 1, the bioactive compounds present in the lyophilized apple are also present in the pulp-derived calli, proving that there is accumulation of these molecules also in cell cultures. In some cases, the amount of the SMs contained in the cell culture is similar to that contained in the starting material.

3.2 Use of salicylic acid as elicitor within callus culture.

One of the several methods adopted to prompt the production of natural compounds within plants cell cultures is the use of elicitors, biotic or abiotic molecules that triggers a signal and thus activates specific pathways within cells. Given this, SA as elicitor was tested, as it is known for its several functions, such as inducing seed germination and flowering, controlling photosynthesis and enzyme activities, therefore stimulating the SM production (Ali et al. 2021).

The addition of SA 50 and 100 mg/L to the medium for the cultivation of pulp-derived calli determined the increase of specific SMs whose amount was comparable or even greater than the one detected in the control and the lyophilized apple (Table 2). Specifically, the addition of SA 100 mg/L induced a major increase in procyanidin B1, gallic acid and ursolic acid. The first two belong to the polyphenol category. These phytochemicals are usually known for their important antioxidant activity and found in much higher concentrations in apple peel (Boyer & Liu, 2004). The third one belongs to the category of triterpenic acid. In particular, ursolic acid has long been investigated for its activity against several types of cancers and its low toxicity after administration (Zafar et. al 2022). These results indicate that pulp-derived cell cultures can be used as bio-factories for the production of valuable quantity of secondary metabolites to be employed for food purposes. Indeed, nutritionally valuable compounds were produced in quantitative amounts that are comparable or better than reference material by optimizing the cell culture with the elicitation.

Since there is a need to increase the intake of plant nutrients in the diet, PCCs can facilitate the shift from an animal-based to a plant-based food, with minimal waste of our planet's primary resources.

Table 2 *The content of metabolites present within pulp-derived calli treated with SA and the control.*

Secondary metabolites(mg) / callus(g)			
Compounds	**Control**	**Treated with 50 mg/L SA**	**Treated with 100 mg/L SA**
Procyanidin B1	0.310±0.044	0.689±0.006***	0.744±0.028***
Procyanidin B2	0.02 ±0.0006	0.041±0.002***	0.040±0.001***
Epicatechin	0.001±0.0003	0.017±0.002***	0.020±0.0002***
Gallic acid	0.102±0.025	0.127±0.004	0.207±0.002**
Ursolic acid	2.910±0.292	2.890±0.288	4.220±0.123**

Values are means ± SE (n = 3) and asterisks denote statistically significant differences of each treatment compared to the control (**p ≤ 0.001; ***p ≤ 0.0001) according to Student's *t*-test.

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Health-promoting properties of food bioactives: the case study of quercetin. Preliminary results.

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The results here described are part of a wider experimental plan aimed at studying the fate of quercetin contained in model systems simulating apple or its derivatives during the gastrointestinal process. To this purpose, the effect of *in-vitro* digestion on quercetin antioxidant activity was assessed. Quercetin's effect on the inhibition of intestinal α-glucosidase in a colorectal adenocarcinoma cell line Caco-2 was also investigated.

Proprietà "*health promoting*" di molecole bioattive presenti negli alimenti: il caso studio della quercetina. Risultati preliminari.

Lo studio, che si inserisce in una più ampia sperimentazione, ha avuto lo scopo di studiare l'effetto sull'attività antiossidante della quercetina contenuta in alimenti modello simulanti una mela o un derivato di mela, del processo digestivo *in-vitro*. Parallelamente è stata anche valutata l'azione di questa molecola bioattiva sull'enzima umano α-glucosidasi nel contesto dell'assorbimento di glucosio a livello intestinale.

Keywords: antioxidant activity, *in-vitro* digestion, quercetin, pectin, antihyperglycemic, α-glucosidase

1. Introduction

Quercetin is a known bioactive compound that can be found in several fruits and vegetables. This polyphenol is recognized to exert several beneficial effects such as antioxidant, anti-inflammatory, and antihyperglycemic activities. Although all these effects have been repeatedly reported, literature results on quercetin's physiological effects sound still controversial and the mechanisms by which it exerts these functions, especially when it is assumed with the diet, are far from being elucidated (D'Archivio *et al.*, 2010). It is a matter of fact that quercetin activity is likely to be affected by the interactions among food components occurring before and during the digestion process (Alongi *et al.*, 2023). Starting from this assumption, the study here described aims to:

A1) Understand the effect of the digestive process on the antioxidant activity of quercetin when contained in a model system mimicking an apple.

A2) Elucidate the ability of quercetin to regulate glucose absorption mechanisms at intestinal level by using Caco-2 cell lines.

The results here reported are part of a wider experimental plan whose main objective is to better understand how and in which extent food composition and structure may affect some mechanisms at the basis of quercetin physiological effects.

2. Materials and Methods

2.1 Antioxidant activity on food model system

The following apple model systems were prepared: (*i*) quercetin-3-glucoside (q-3-glu) in sugars solution that was obtained by dissolving q-3-glu at a concentration of 0.33 mg/mL, approximated to the total phenolic content reported for *Golden delicious* apples (Alongi *et al.*, 2018), in a solution containing 15 mg/mL glucose, 30 mg/mL fructose, and 4 mg/mL sucrose, mimicking apple sugar concentration; (*ii*) pectin in sugars solution, which was obtained by dissolving pectin at a concentration of 2.8 mg/mL (Baker, 1997); (*iii*) q-3-glu and pectin in sugars solution was obtained by adding q-3-glu in the sugars-pectin solution described in (*ii*).
In-vitro digestion was carried out according to the INFOGEST protocol (Brodkorb *et al.*, 2019), and subsequent analyses were carried out on the bioaccessible fraction. The antioxidant activity was assessed by the DPPH method (Manzocco, Anese and Nicoli, 1998). Data are expressed in milligrams of Trolox Equivalents per litre (mg_{TE}/L).

2.2 Inhibition of α-glucosidase in Caco-2 cells

Caco-2 cells were cultured on Transwell inserts as reported by Kan et al., 2021. The concentration tested were q-3-glu 2 µM and 25 µM. Acarbose 50 µM was used as a positive control, and sucrose 75 mM without stimuli as a negative control. To evaluate the effect of α-glucosidase, sucrose concentration in the culture media was assayed at different times (0.5, 1, 4 and 24 h). Measurements were carried out using the MEGAZYME Glucose/Fructose/Sucrose HK assay kit (NEOGEN Europe Ltd) with a BIOTEK Synergy H1 plate reader (Agilent Technologies). All the concentrations of q-3-g and Acarbose used were previously tested with CyQUANT LDH assay (Thermo Fisher Scientific, Italy), and none of them significantly affected Caco-2 cell viability.

3. Results and Discussion

3.1 Antioxidant activity of apple model systems

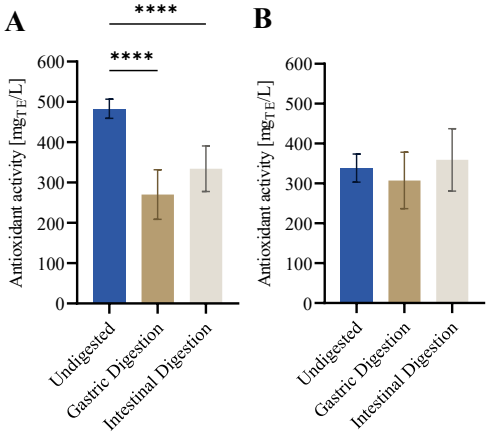

Figure 1: Antioxidant activity of food model system containing: (**A**) quercetin-3-glucoside in sugars solution; (**B**) quercetin-3-glucoside and pectin in sugars solution subjected or not to *in-vitro* gastric and intestinal digestion.
Data expressed as mean ± SD, n = 2 biological replicates, statistically significant results are labelled (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p \leq 0.001$).

Figure 1A shows the antioxidant activity relevant to food model systems before and after *in vitro* digestion. The only contributor to the antioxidant activity was quercetin-3-glucoside since sugars and pectin did not show any antioxidant activity (data not shown). The gastric process seems responsible for a loss of about 45% of the original antioxidant properties. No significant changes were further detected as a consequence of the intestinal digestion. When pectin was present (Figure 1B), a lower antioxidant activity was measured before *in vitro* digestion. It is likely that pectin generated a network able to embed quercetin, thus reducing its reactivity towards DPPH in the undigested sample. Conversely, the presence of pectin did not affect the antioxidant activity in digested samples. These results suggest that the pectin shell is modified by the gastrointestinal events, allowing quercetin to exert its bioactivity. Trials are in progress to elucidate the fate of pectin during digestion in order to confirm this hypothesis.

3.2 α-Glucosidase inhibitory activity of quercetin-3-glucoside

Figure 2 shows the percentage of residual sucrose in the culture media of Caco-2 cells treated with different concentrations of q-3-glu. Acarbose, which is a known α-glucosidase inhibitor, was used as positive control. The higher amount of residual sucrose in the culture media observed for the sample containing q-3-glu 2 µM suggest the ability of quercetin in reducing sucrose enzymatic hydrolysis. This trend is similar to that observed with acarbose. Sample containing q-3-glu 25 µM showed a negligible effect, suggesting that quercetin could inhibit α-glucosidase only in a given concentration range.

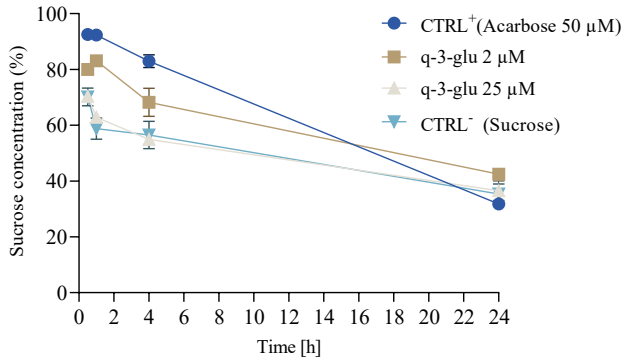

Figure 2: Percentage of residual sucrose concentration in culture media of Caco-2 cells treated with 2 and 25 µM of quercetin-3-glucoside over a time-course of 24h.
Preliminary data expressed as mean ± SD, n = 1 biological replicate.

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Production, Composition and Sensory Characterization of New Flavoured Oils: Focus on Sustainability

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The activities of this PhD project concern, firstly, the characterization of specific tomato and hemp by-products and the application of sustainable technologies to obtain from them stable raw materials/ingredients which can be involved in the production of flavoured oils. In particular, a tomato pomace has been dried out with a sustainable approach and essential oils have been extracted from a hemp biomass. Secondly, vegetable oils (cold pressed hemp seed oil and virgin olive oil) have been flavoured with such vegetable matrices, as well as by using commercial powders made of tomato and orange by-product.

Produzione, composizione e caratterizzazione sensoriale di nuovi oli aromatizzati: focus sulla sostenibilità

Le attività del progetto riguardano, in primo luogo, la caratterizzazione di specifici sottoprodotti del pomodoro e della canapa e l'applicazione di tecnologie sostenibili per la produzione, da queste matrici, di ingredienti stabili che possano essere utilizzati nella formulazione di oli aromatizzati. In particolare, il sottoprodotto del pomodoro è stato essiccato mediante un approccio sostenibile mentre dalla biomassa di canapa è stato ottenuto un olio essenziale. Successivamente, oli vegetali (olio vergine di oliva e olio di semi di canapa spremuti a freddo) sono stati aromatizzati con tali matrici e con polveri commerciali a base di pomodoro e sottoprodotto di arancia.

Key words: flavoured vegetable oils, hemp, tomato, orange, by-products, valorization.

1. Introduction

This contribution reports the main results of the following activities:

(A1) the characterization of specific hemp and tomato by-products and the applications of sustainable technologies, in particular non-thermal drying and distillation to promote the durability of these matrices.

(A2) the production of flavoured vegetable oils and their evaluation in terms of quality related parameters, such as free acidity, sensory profile, volatile compounds, oxidative stability, cannabinoids content, as well as the assessment of consumers' perception, liking and acceptability.

2. Materials and Methods

(A1) The tomato pomace, made of skin and seeds, has been dried out using a non-thermal drier prototype, which functions with a flux of air at room temperature, able to mix and dry these vegetable matrices inside of its two chambers. Humidity (with a gravimetric approach) and water activity (Aqualab, Decagon Devices Inc., Pullman, USA) have been assessed. It has been chosen to test the action of the prototype with two different settings: with and without the use of compressed air. For what concerns the production of hemp essential oils, 20 g of a specific hemp by-product, derived from the industrial production of cannabidiol (CBD) by extraction, has been hydrodistilled using a lab distillatory prototypal system.

(A2) Tomato flavoured olive oil has been produced at lab scale by co-malaxation of olives with tomato pomace using the lab scale mill Abencor® (MC2 Ingeniería y Sistemas S.L, Sevilla, Spain), while hemp flavoured hemp seed oil has been obtained by the addition of hemp essential oil to a commercial cold pressed hemp seed oil. Free acidity (EU regulation 2022/2104) and volatile compounds profile (analytical conditions described in Aparicio-Ruiz *et al*., 2023) have been evaluated for both the flavoured oils and the related control samples, while the oxidative stability (Rancimat®) has been assessed only for the hemp seed one, such as the cannabinoids content and the peroxide values. A commercial orange powder, made of orange pomace and another marketed tomato powder have been added during the malaxation to produce a flavoured olive oil whose composition is going to be analysed. In addition, two other flavoured olive oils have been produced by infusion (for 72 h), favoured by the use of a sonic bath (Ultrasonic Cleaner 2200 S3, Milan, Italy), of canned tomatoes and an orange by-product manually produced from juicing. Consumer tests on the abovementioned hemp-seed oils have been carried out to assess the sensory profile (CATA, Check All That Apply) and the consumers' perception of specific attributes (JAR, Just About Right scale) as well as their liking (9-points hedonic scale) and preference (2-AFC, 2-alternative-forced-choice). Moreover, a joint sensory-biometric analysis is ongoing to assess their liking, specific attributes intensity (JAR scale) and how information regarding the assessed vegetable oils (tomato flavoured olive oil, orange flavoured olive and hemp flavoured hemp seed oil) production and sustainability may influence the perception.

3. Results and Discussion

3.1 (A1) Determination of a_w in the dried tomato pomace; yield assessment in the hemp essential oil

It has been chosen to stop the drying process of the tomato pomace after 3h of treatment since the a_w has reached levels around 0.4 and 0.5 for the batches dried with and without the use of compressed air, respectively. The levels reached during this study are sufficient since around these values of a_w the growth of most microorganisms and moulds is very limited (Labuza, 1980).

Concerning the production of hemp essential oils, a prototypal distillation system has been used and its collection, as a flavouring technique, is now under the evaluation of the UNIBO Knowledge Transfer Office for a possible patent. The obtained yield (0.19%) of essential oil is coherent with the literature (Zheljazkov *et al.,* 2020).

3.2 Flavoured vegetable oils: quality parameters

The produced oils have been tested to evaluate their quality; several sensory and compositional parameters are now under investigation (e.g. consumer tests to evaluate preferences, total phenols content, carotenoids content, …). Free acidity has been evaluated for each vegetable oil sample, including the test samples, and each of them has shown values below the limits in the regulations and standard (Codex Stan 210-1999 and EU regulation 2022/2104), with values ranging from 0.17 ± 6.7*10^{-5} % to 0.22 ± 0.001 % of oleic acid for olive oils, and 1.3 ± 0.1 mg KOH/g of oil for hemp seed oils.

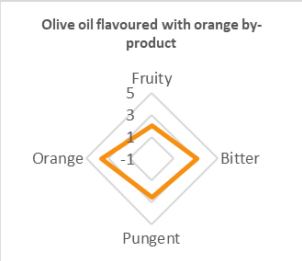

Figure 1 *Radar graph: intensity of the main and secondary positive attributes of a produced olive oil flavoured with orange*

Flavoured olive oils are not included in the commercial categories extra virgin, virgin or lampante, since they are "dressings based on olive oil": they do not meet the definition stated in EU regulation 2022/2104; the levels of free acidity, in this case, provides anyway information regarding their quality.

From preliminary results the flavoured olive oils appear to be of high quality with peculiar sensory notes, assessed by descriptive analysis, derived from the added vegetable matrices (example in Fig. 1).

Preliminary semi-quantitative analysis of the volatile compounds profiles has been carried out and for what concerns hemp seed oil and flavoured hemp seed oil, it has been possible to highlight some differences related to the presence of the essential oils from hemp by-product. In particular, from the heat map shown in Fig. 2, some terpenes (monoterpenes and sesquiterpenes) appear at higher concentrations in the flavoured hemp seed oil.

These compounds, such as β-myrcene, linalool, limonene, α-terpineol and β-caryophyllene are related to fruity, floral, sweet and sensory notes and for which anti-inflammatory and antioxidant properties have been found (Surendran *et al*., 2021; Tura *et al.*, 2023).

In addition, from the preliminary results of consumer tests, the hemp-flavoured hemp seed oil appeared to be more liked, considering its aroma (mean score = 6.7) rather than the control one (5.8). Moreover, participants were asked to identify their favourite sample in a 2-AFC test, which significantly results the flavoured one ($p = 0.0033$, $\alpha = 0.05$). These results suggest that the aroma of the EOs, extracted from the hemp by-product, might be a driver of preference such oils, thus positively affecting the subjects' liking. This finding, among others, will be verified with the further consumer tests data elaboration.

The same evaluations will be also carried out for the other produced flavoured oils.

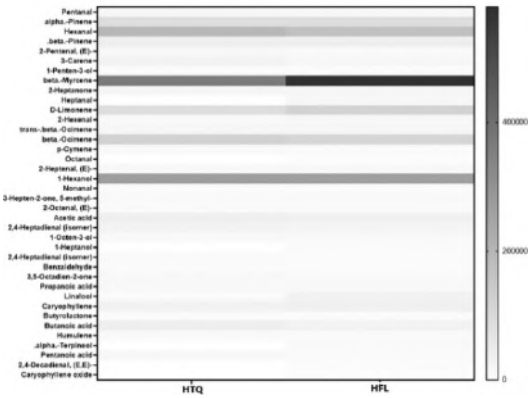

Figure 2 *Heat map comparing the content of the volatile compounds in the two hemp seed oils (HTQ, control sample of hemp seed oil, and HFL, flavoured hemp seed oil).*

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"Green" Technologies in the Supply Chain of Agri-Food Company

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This PhD thesis research project involves three experimental plots of Soreli kiwifruit vineyards, irrigated with three different water regimes, and the plants subjected to the various irrigations are monitored by an IoT-TT Spectrum which provides data in real time, and a Soil sensor to determine the moisture content. Fruit quality analysis for weight loss, total soluble solid content, flesh color, flesh firmness, titratable acidity, ascorbic acid, total polyphenols, and flavonoids is conducted to correlate the productivity rate to amount of water availability. Overall, this study demonstrates that excessive or limited irrigation regimes adversely affect plant health based on vegetation indices and fruit quality.

Recupero di alcuni metabolici microbici da soluzioni modello per osmosi inversa

Questa proposta di tesi di dottorato coinvolge tre settori sperimentali di kiwi Soreli, irrigati con tre diversi regimi idrici, e le piante sottoposte alle diverse irrigazioni sono monitorate da uno spettro IoT-TT che fornisce dati in tempo reale, e un sensore del suolo per determinare il contenuto di umidità. L'analisi della qualità della frutta per la perdita di peso, il contenuto solido solubile totale, il colore, la consistenza, l'acidità titolabile, l'acido ascorbico, i polifenoli totali e i flavonoidi viene condotta per correlare il tasso di produttività alla quantità di disponibilità di acqua. Nel complesso, questo studio dimostra che regimi di irrigazione eccessivi o limitati influenzano negativamente la salute delle piante sulla base degli indici di vegetazione e della qualità dei frutti.

Key words: IoT, TT-Spectrum, Soreli kiwifruit, Leaf chlorophyll content, Fruit quality, Irrigation.

1. Introduction

In accordance with the PhD thesis project previously described (Rolle et al., 2022), this poster reports the main results of the first two activities concerning:

(A1) the determination of vegetation indices (NDVI, CVI, LNBI, and AI) and soil moisture content obtained through the analysis of data provided by IoT-TT Spectrum and Soil sensor; these are important indicators of the physiological status of kiwifruit plants and can be used to assess plant health and growth.

(A2) the assessment of the quality fruits through analysis of the chemical-physical parameters and bioactive compounds; These analyses aim to assess how the water deficit affects the productivity of the plant and the quality of the fruit being harvested.

2. Materials and Methods

2.1 Experimental design

In the summer of 2022 three experimental plots of "Soreli" kiwifruit vineyards at the "Tre Colli" farm, situated in Cisterna Campoleone (Velletri, Rome, Italy), were selected and irrigated with different volumes of water; 100% or full irrigation and deficit irrigation at 80% and 60% relative to the full irrigation approach.

2.2 TT Spectrum and soil sensors

Three IoT-TT Spectrums and three soil sensors for each treatment were used to obtain real-time observation of physical and biological parameters of the leaves and on soil moisture. TT Spectrum collects light reflectance data in 12 spectral bands, 610+, 680+, 730+, 760+, 810+, 860+ nm, 450*, 500*, 550*, 570*, 600*, 650* (* ±20 nm + ±40 nm). For further processing, four vegetation indices, Normalized Difference Vegetation Index (NDVI) (Tucker, 1979), Chlorophyll Vegetation Index (CVI) (Vincini et al. 2008), Leaf Nitrogen Balance Index (LNBI) (Fan et al. 2022) and Anthocyanin Index (AI) (Gitelson et al. 2001) are evaluated from TT Spectrum data.

2.3 Kiwi fruit analysis

Soreli kiwi fruits were collected and transported to the Postharvest Laboratory of DIBAF (University of Tuscia, Viterbo, Italy). Kiwifruits were stored for 24 h at room temperature and were then cooled to 1 ± 0.5 ◦C with 85 ± 5% RH in normal atmosphere with ethylene absorber. All analyses were performed at harvest time and after every 15 days of cold storage. At harvest and before storage the same 20 kiwifruits were weighed using a digital balance (Adam Equipment Co., Ltd., Milton Keynes, UK), to monitor weight loss during cold storage. Total soluble solid content (SCC) was measured on the fresh kiwifruit juice using a digital refractometer (ATAGO, Palette PR-32, Tokyo, Japan) and expressed as °Brix (%). Flesh colour was measured on peeled fruits using a Minolta colorimeter to evaluate the chromaticity values L*, a*, and b*. Flesh firmness was evaluated with a destructive method using a digital penetrometer (Mod. 53205; TR Turoni snc, Forlì, Italy), and with non-destructive method with an Instron

Universal Tasting Machine. Titratable acidity was measured on the flesh juice according to the protocol of Grasso et al. (2022). Total phenols (TP) and flavonoids (TF) content was determined as reported by Grasso et al. (2022). Ascorbic acid (AA) content was assessed according to Grasso et al. (2022). Chlorophyll a, Chlorophyll b and β-Carotene in the sample were analysed as described by Goffi et al., (2017).

2.4 Statistical Analyses

All results are expressed as the means ± standard deviation (SD). Statistical significances between different maturity stages of the fruit of different orchards and storage time were analysed by two-way analysis of variance (ANOVA), and Tukey's test at 5% level was calculated to compare differences between means. Differences at $p < 0.05$ were considered significant and are indicated with different letters.

3. Results and Discussion

3.1 Effects of Overwatering or Underwatering on Kiwifruit Plants

In Figure 1, we present the TT-Spectrum output for the main vegetation indices for each individual, based on the reflectance data from various reported bands (450 to 860 nm). The results demonstrate that different levels of soil moisture availability for kiwifruit plants lead to distinct ecophysiological responses (Figure 1). The soil moisture data revealed that each individual plant received different levels of water availability, resulting in varying health, growth, and productivity conditions. For example, Tree 76226005 received lower irrigation during the early season and maximum water availability during the dry period, which resulted in a very high NDVI and CVI, lower AI, and a balanced leaf nitrogen level. In contrast, Tree 76226000 received full irrigation during the early season, and underwatering during the dry period did not show high foliage health based on the results of the aforementioned indexes (Figure 1).

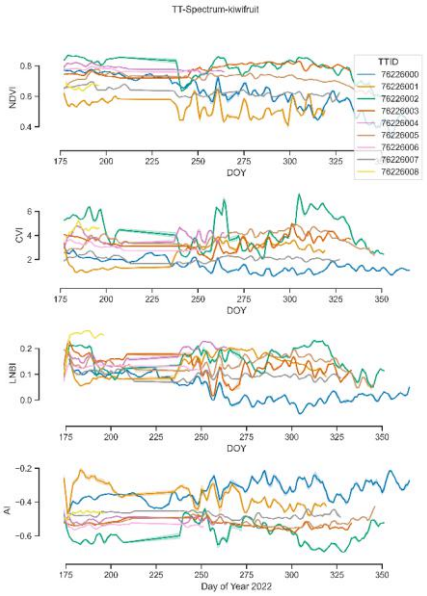

Figure 1 *The TT-Spectrum output for the main vegetation indices (NDVI, CVI, LNBI, and AI) for each individual, using reflectance data from different reported bands*

1.2 Effects of water deficit on Soreli kiwifruit Chemical-Physical Parameters and Bioactive Compounds

The Brix° in stored fruits exhibited a notable increase over time across all treatments while the percentage of weight loss in fruits rose over time and no significant differences were observed between the various irrigation treatments. However, fruits subjected to full irrigation demonstrated lower sugar levels compared to those under deficit irrigation. The firmness decreased progressively for all fruits harvested during cold storage for all irrigations. Overall, the different samples had a uniform trend in terms of softening during the storage. For the colour, L*, a* and b* parameters at the time of harvesting were highest in fruits irrigated with 100% of water compared to fruits irrigated with 80% and 60% of water, while the results showed a decreasing and homogeneous trend until the end of the study without significant differences between the different irrigations. The changes in bioactive compounds content during ripening of kiwifruits do not appear to be significant between the irrigations.

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Application of an eco-sustainable technology: use of direct and photodynamic UV light for the microbial decontamination on food industries

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In the first part of the project, a qualitative screening was done to evaluate the inactivation capacity of the blue LED lamps (BL) against several microbes (*E. coli*, *S. aureus*, *L. monocytogenes*, *S.* Enterica, *P. fluorescens, B. subtilis* and *S. cerevisiae*) testing different doses. Secondly, a quantitative assessment of the direct decontamination capacity was done in solid and liquid media testing different conditions (doses, treatment time, lamp's voltage). Thirdly, the biofilm-forming capacity of microbes on different surfaces was analysed. Biofilms were treated with BL and observed by CLSM. Finally, preliminary activities for the photoactive materials tests were done.

Applicazione di una tecnologia ecosostenibile: utilizzo della luce UV LED diretta o fotodinamica per processi di decontaminazione microbica nell'industria alimentare

Nella prima parte del progetto è stato condotto uno screening qualitativo per valutare la capacità di inattivazione microbica delle lampade LED a luce blu (LB) erogando diverse dosi di luce. È stata poi valutata la decontaminazione diretta operata dalla LB, considerando diversi parametri (dose, tempo, voltaggio delle lampade), e la capacità di formazione di biofilm su diverse superfici. I biofilm sono stati sottoposti a trattamento con BL. I danni cellulari sono stati osservati con CLSM. Per la sperimentazione con i materiali fotoattivabili, sono state svolte prove sperimentali per il set-up dei test con LB.

Key words: Blue LED light, photodynamic inactivation, biofilm, microbial decontamination.

1. Introduction

In accordance with the PhD thesis project, here are reported the main results of the PhD project:
A1) Assessment of the germicidal effect on planktonic cells and biofilm
A2) Tests with photoactive materials

2. Materials and Methods

The qualitative screening was done by streaking microbes on an agar plate and testing the plate under BL at 405, 420 and 450 nm for 1h at 2, 5 and 10 cm from the lamp to modulate the dose (Cheng et al., 2020). Half of the plate was covered as a control. Before each test, irradiance (I) was measured using a probe and this value was used to calculate the dose (D), following Equation 1:

$$D = I \times t \quad (1)$$

Where D is the dose (J/cm^2), I is the irradiance (W/cm^2) and t is the treatment time (s) (Ghate et al., 2013).
The direct inactivation assessment was done in solid and liquid substrates. In the first case, each tested microbe was serially diluted and plated in an agar plate with the drop plate method (Herigstad et al., 2001). Also in this case, half of the plate was covered as a control. In the direct inactivation assessment in liquid media, a 24-well microtiter plate was used. 1 mL of microbe culture in PBS was inserted in each well and half of the plate was covered as a control. The microbial inactivation was monitored every 15 min for two hours. Tests were done by setting the lamp at 10V and 5V, based on the microbes' response. For qualitative and quantitative assessment, the temperature was measured during the tests. All the data were expressed as log N/N_0, where N is the final microbial population after the BL treatment and N_0 is the initial microbial population.
The biofilm formation capacity was initially assessed with a screening in 96-microtiter wells (Stepanović et al., 2007) and then biofilm formation was tested on glass, stainless steel, and Teflon coupons for 48 hours, 7 and 15 days (Li et al., 2018). Biofilms were treated with BL and the microbial inactivation was monitored by microbial cell count, using the drop plate method, by recovery of the cells before and after the treatment. Damages at biofilm structure and morphology were evaluated by CLSM.
For the photoactive materials, preliminary tests were done to set up the BL treatments since the surface is not homogeneous.

3. Results and Discussion

3.1 Qualitative screening

The qualitative screening of the mentioned microbes evidenced that the light-sample distance of 2 cm was not optimal for the BL treatment since it increased the dose and so the final temperature in 60 min reached > 50°C in all of the cases with some exceptions exceeding >70°C. This screening evidenced conditions where the microbial inactivation occurred due to T effect (Figure 1. a.) or where was T not influenced (Figure 1. b.). Furthermore, it is underlying how the wavelength effect is dependent on the microbe that is used.

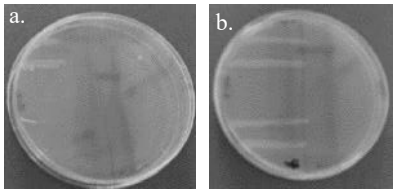

Figure 1 *a. L. monocytogenes*, *S.* Enterica 2 cm 405 nm; *b. L. monocytogenes*, *S.* Enterica 5 cm 405 nm.

3.2 Quantitative assessment: cells

As evidenced by quantitative screening, the 2 cm lamp-sample distance was not optimal. Therefore, the quantitative tests on solid media proceed with light-sample distances of 5 cm and 10 cm. Tests evidence that at 5 cm almost all the microorganisms were completely inactivated with no temperature effect with using all the wavelengths. An exception was for *L. monocytogenes* and *P. fluorescens*, which were affected by the high temperature (ca. 48°C). Indeed, at 10 cm microbes follow different inactivation based on the wavelength that was used and the emitted doses. For instance, *S. aureus* was less affected by 450 nm (-1.61±0.70, D=198 J/cm^2, T_{60}=36.5°C), while at 405 and 420 nm the inactivation was -4.76± 0.87 (D=144 J/cm^2, T_{60}=34.7°C) and -3.02±0.48 (D = 162 J/cm^2, T_{60}=37.5°C), respectively. *L. monocytogenes* was reduced up to -1.91 ± 0.28 (D=216 J/cm^2, T_{60}=39.4°C), -7.34 ± 0.30 (D=198 J/cm^2, T_{60}=37.1°C) and -4.31 ± 0.43 (D=198 J/cm^2, T_{60}=37.7°C) at 450, 405 and 420 respectively. In general, all microbes tested were less susceptible to 450 nm, with some exceptions. For instance, *S.* Enterica shows poor inactivation at all the wavelengths (max inactivation -1.31 ± 0.13 at 420 nm, D= 180 J/cm^2, T_{60}=37.1°C).

3.3 Quantitative assessment: cells in suspension

Tests in the liquid substrate were done on *E. coli*, *S.* Enterica, *L. monocytogenes* and *S. cerevisiae*. *E. coli* irradiated with 405 nm (10 V) was reduced up to -4.81±0.95 in 90 min (T_{90} =39.9; Dose = 280 J/cm^2), -3.81±0.47 at 420 nm (T_{90} =39.4°C; Dose = 280 J/cm^2) and -1.82 at ±1.04 at 450nm (T_{90} =38.7; Dose = 280 J/cm^2), while the dark controls remain almost constant. *S.* Enterica was more resistant to BL. In fact, at 405 the reduction was -2.08 ± 1.10 after 120 min (T_{120} =43.5; Dose = 360); however, at 420 nm and 450 nm, the reduction was < 1 log cfu/mL. *L. monocytogenes* showed better results by setting the light voltage at 5V. Using the 405 nm lamp, it decreased up to -3.15 ±0.47 after 120 min (T_{120} =31.6; Dose = 324 J/cm^2). Also in this case, the lower effect was seen for 450nm lamp, -1.08±0.01 after 120 min (T_{120} =31.5; Dose = 324). *S. cerevisiae* after 120 min decreased up to -1.23±0.26 (T_{120} =40.4; Dose = 396 J/cm^2), -1.41±0.07 (T_{120} =44.1; Dose = 360 J/cm^2) and -1.43±0.23 (T_{120} =40.5; Dose = 432 J/cm^2) for 450, 405 and 420 nm, respectively. However, the dark control decreased after 120 min -1.12±0.26, -0.86±0.19 and -1.56±0.37 at 450, 405 and 420 nm, respectively. This was probably because the temperatures reached were 33.4, 32.1 and 34,5 °C at 450, 405 and 420 nm, respectively. Also, the BL has a different efficacy on microorganisms, depending on the microbial species. Moreover, by CLSM observations of biofilms, the BL affected the biofilm's structure formation and produced cellular damage on planktonic cells (study in progress).

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Use of meta-omics approaches for characterization of microbiota isolated from different ecological niches

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In the present work, the first two activities of the PhD thesis are described. Firstly, pomegranate microbiota was investigated trough culture-dependent approach and potentially pro-technological microorganisms were isolated and identified. Secondly, fermentation process of pomegranate juices and seeds by microorganisms previously isolated were set up. New large-scale consumption foods were designed, on laboratory scale, and characterized.

Approcci meta-omici per la caratterizzazione del microbiota in diverse nicchie ecologiche

Nel presente lavoro sono descritte le prime due attività del progetto di tesi di dottorato. Il microbiota del frutto del melograno è stato studiato mediante approccio coltura-dipendente e i microrganismi potenzialmente pro-tecnologici sono stati isolati ed identificati. Successivamente, succhi e semi di melagrana sono stati fermentati con i microrganismi precedentemente isolati al fine di progettare, su scala di laboratorio, nuovi alimenti di largo consumo e caratterizzarli.

Key words: pomegranate, yeasts, by-products, granola, cider.

1. Introduction

In accordance with the PhD thesis project previously described in Proceedings of the 26th Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology (Rolle et al., 2022), this poster reports the main results of the first two activities concerning:

(A1) isolation and identification of potentially pro-technological microorganisms from pomegranate juice (PJ) and seeds (PS), able to ferment the same matrix;

(A2) exploitation, through fermentation, of pomegranate juice and seeds, to develop, on pilot plant, new large-scale consumption foods (soft drink from PJ and seeds flour from PS, to be added as an ingredient), in order to better meet consumer's expectations.

2. Materials and Methods

2.1 Isolation and identification of pro-technological microorganisms from PJ and PS

PJ and PS from fresh arils were used as source of isolation of pro-technological microorganisms after spontaneous fermentation. To obtain a substrate for spontaneous fermentation of seeds, a semi-solid matrix was prepared adding tap water to PS (seeds:water ratio of ca. 4:1). Both juice and water-seeds mixture were incubated at 30 °C for 16 h. Spontaneously fermented juice and seeds were then analysed to isolate lactic acid bacteria and yeasts, using serial dilutions method. After incubation of plates, only yeast colonies were found. Therefore, yeasts were isolated by picking up colonies from the Sabouraud Dextrose (SD) agar plates inoculated with the highest diluted samples. Yeasts DNA was extracted using the Wizard® Genomic DNA purification kit, according to the manufacturer's instructions. Yeasts were biotyped by RAPD-PCR analysis singly using the primers M13m and RP11 (Del Bove et al., 2009). Yeasts were identified upon partial sequencing of 26S rRNA gene, amplified using the primers NL-1 and NL-4 (Kurtzman and Robnett, 1998). PCR products were sequenced by Macrogen Europe BV and the DNA sequence homology (higher than 99%) was determined through pair-wise sequence alignments, using BLAST within the NCBI nucleotide collection database.

2.2 Fermentation of PJ and PS and characterization of the fermented products

Commercial pasteurized 100% natural PJ were used in the second part of this study. Commercial PJ differed in sugar content (8.9 and 12.2 for low sugar (LS) and high sugar (HS) respectively). PS were obtained from chemically sterilized arils by squeezing them through a lab blender mixer in a stomacher bag with lateral filter for three cycles of 3 min each.

Yeast strains isolated from pomegranate matrices were singly used as starters for fermenting PJ and PS (seeds:water ratio of ca. 4:1). When used for fermentation, yeasts were cultivated at 30 °C (24 h or 48 h, respectively for PJ or PS) in SD broth, harvested by centrifugation (10000×*g*, 10 min, 4 °C), washed twice with 50 mM sterile potassium phosphate buffer (pH 7.0), re-suspended in juices samples or in sterile distilled water and used to inoculate PJ or PS, respectively (initial cell number corresponding to ca. 5.5 log cfu/g in juices and 7.0 in seeds). A commercial yeast (CY) was used as a control to ferment PJ. All the juices were fermented at 15 °C for

seven days, to obtain pomegranate cider. In addition, PS were spontaneously fermented without the addition of starters at 30 °C for 24 h. At the end of fermentations, pomegranate cider was stored at 4 °C; PS were dried in an oven at 60 °C overnight, ground through a coffee mill and the flours obtained were used as an ingredient for production of *granola* snack. Cell density of presumptive yeasts, chemical analyses, determination of antioxidant activity were carried out along all the production process and at the end of it. Moreover, volatile compounds produced during ciders fermentation will be analyzed.

3. Results and Discussion

3.1 Isolation and identification of pro-technological microorganisms from PJ and PS

Pomegranate microbiota was dominated by yeasts, even after spontaneous fermentation. This microbial group, probably environment-borne, typically contaminates fruit peels (Kalia and Gupta, 2006). Notwithstanding the use of selective or elective culture media for specific groups of bacteria, we did not find bacteria at detectable cell density, even after spontaneous fermentation. This could be due to the low pH and the presence of many bactericidal compounds (e.g., hydrolysable tannins, ellagitannins, gallotannins, anthocyanins and flavonols) in the pomegranate matrices (Howell and D'Souza, 2013; Reddy et al., 2007). Therefore, we isolated yeasts from both spontaneously fermented PJ and PS. After biotyping through RAPD-PCR, twelve isolates were grouped into four clusters (I-IV). Clusters I, II and III included isolates from PJ. The IV cluster only grouped isolates from PS. Isolates representative for each cluster (namely J-L1, J-L5, J-L6 and S-L1 from juice and seeds, respectively) were subjected to molecular identification. All the isolates were identified as *Hanseniaspora valbyensis*, a non-conventional yeast often found as one of the microbial drivers of balsamic vinegar and cider fermentations (Bellut et al., 2018). Strains of *H. valbyensis* were singly used as starter for driven fermentation of PJ and PS.

3.2 Fermentation of PJ and PS and characterization of the fermented products

The four representative autochthonous strains of *H. valbyensis* were able to drive the fermentation of pomegranate matrices, causing modifications of the chemical composition. Among the four autochthonous yeasts, we selected *H. valbyensis* J-L6 and S-L1 for the fermentation of juices and seeds respectively, because besides being able to grow in the pomegranate matrices, they caused the best increase in mineral content. Therefore, we tried to design a pomegranate cider from PJ and a fortified snack (*granola*) supplemented with PS each one fermented with a different strain (S-L1 for PS, J-L6 for cider) of *H. valbyensis*. After three days of fermentation of PJ, cell density of moulds and yeasts increased significantly ($p < 0.05$) in all the thesis (LS-CY, LS J-L6, HS-CY, HS J-L6); however, the highest increase (about three logarithmic cycles) was observed in HS e LS juices fermented by *H. valbyensis* J-L6. No significant differences ($p > 0.05$) of cell density of yeast were observed after seven days with respect to day 3, except for LS and HS juice fermented by commercial yeast, wherein cell density increased significantly ($p < 0.05$). Despite this noticeable growth, yeast cell density was still significantly higher in PJ fermented by J-L6. Chemical analyses, including mineral composition, sugar (glucose, fructose, sucrose), alcohol and acetic acid concentration, profile of volatile compounds, and antioxidant activity of ciders will be investigated. PS flour from unfermented seeds and from seeds fermented by *H. valbyensis* S-L1, were used as additional ingredient for the production of *granola* snack (G-US and G-FS respectively). Compared to *granola* control (G-C) obtained without adding pomegranate seeds flour (PSF), the use of PSF, as an additional ingredient of *granola*, increased *in vitro* antioxidant activity of the snack. The expected contents of calcium, iron, potassium, and zinc were higher in G-FS and G-C, compared to G-US. Finally, we expect that the two *granola* snacks fortified with PSF would have lower (ca. 15%) energetic values than control *granola*. Fermentation of seeds also contributed to significantly improve the overall acceptability of *granola* snacks with seeds flour, making it more similar to the control and more appreciated than *granola* containing unfermented seeds.

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Advanced Emulsions as Bioactive Compound Carriers for Functional Food Design: Technological and Nutritional Aspects

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Four types of emulsions (i.e., single, cold filled-gel, hot filled-gel, and Pickering) were produced and characterized to develop carriers for polyphenols extracted from olive-vegetative water. Central Composite Designs were applied to study the effects of different emulsifier types and concentrations, and oil phase percentages. After the technological characterization of all the samples, Response Surface Methodology was applied to obtain optimized emulsion formulations for the future encapsulation of polyphenols and food enrichment applications.

Emulsioni avanzate come sistemi di trasporto di composti bioattivi per lo sviluppo di alimenti funzionali: aspetti tecnologici e nutrizionali

Quattro tipologie di emulsioni sono state studiate come possibili *carrier* per polifenoli derivanti dalle acque di vegetazione delle olive: singole, gelificate a freddo, gelificate a caldo e Pickering. L'applicazione di *Central Composite Designs* ha permesso di studiare l'effetto delle differenti tipologie di emulsionanti, della concentrazione di emulsionante e di diverse percentuali di fase oleosa. Attraverso la metodica delle superfici di risposta, sono state ottimizzate le formulazioni delle emulsioni da utilizzare in futuro per l'incapsulamento di polifenoli e lo sviluppo di alimenti arricchiti.

Keywords: Pickering emulsion, filled-gel emulsion, single emulsion, Design of Experiment, Response Surface Methodology.

1. Introduction

The PhD project is about the development of lipid carriers for natural polyphenol extract obtained from olive-vegetative water, for future food reformulation to promote sustainability and healthiness. Here, the main results derived from the first activities of the project are shown:

(A1) Design of different types of advanced emulsions by applying Design of Experiment (DoE) techniques to study the main and interaction effects of type of emulsions, concentration of emulsifiers, and concentration of corn oil, and characterization of emulsions for the most relevant technological parameters.

(A2) Optimization of the different emulsion formulations by applying the Response Surface Methodology (RSM).

2. Materials and Methods

Four different oil-in-water emulsions were studied: single, cold filled-gel, hot filled-gel, and Pickering emulsions. By applying a face-centred central composite design (Design Expert, v. 10.0.0.3, Stat-Ease Inc., MN, USA), thirteen runs for each kind of emulsion were developed and characterized. Lecithin (Le; 0.5-1.5-2.5% in oil) and citrus fibres (CF; 4-6-8% in water) were tested as emulsifiers for single and cold filled-gel emulsions, respectively; chickpea protein isolate (CP; 8-12-16% in water) was used as emulsifier in both hot filled-gel and Pickering emulsions. Corn oil was studied at 15-37.5-60% levels in all the emulsions. After mixing the emulsifier, the water and the oil phase were homogenized with a high-shear homogenizer (UltraTurrax T25, IKA, Germany) at 21000 rpm for two cycles of 30 s, with a resting phase of 30 s. All samples were analysed for pH (SevenEase, Mettler Toledo S.p.A., Italy), particle size distribution (Mastersizer 3000, Malvern Panalytical, UK), apparent viscosity (MCR 102 rheometer, Anton Paar, Austria), and stability at 4°C for 14 days. Moreover, optical (mod. B1000 with digital camera, Optika Microscopes, Italy) and confocal laser scanning (Nikon A1, Nikon, Netherlands) microscopy observations were performed. All the results were statistically analysed with a Multifactor Analysis of Variance (MANOVA) followed by the Least Significant Difference (LSD) test to identify the significant main and interaction effects (i.e., A. emulsion type; B: emulsifier concentration; C: corn oil concentration; AB, AC, BC: two-way interactions). The Pearson correlation matrix (Statgraphics Centurion 18, v. 18.1.13) was also studied, to identify significant correlations between couples of variables. At last, RSM (Design Expert, v. 10.0.0.3, Stat-Ease Inc., Minneapolis, MN, USA) was applied for the optimization of the four emulsion formulations.

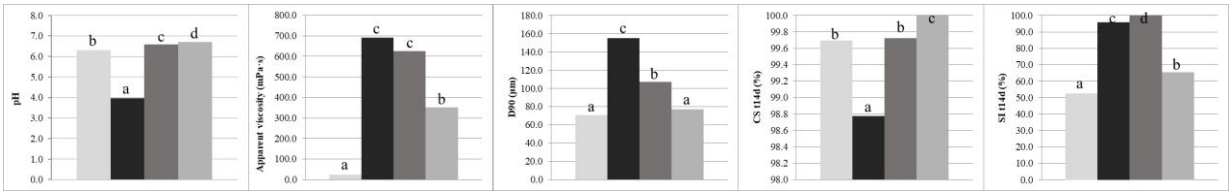
3. Results and Discussion

3.1 Main and interaction effects evaluation

Microscopy observations confirmed the different structure of the advanced emulsions, revealing the adsorption of CP at the oil droplet interfaces in Pickering emulsions, while a 3D-network was created by gelled CP in hot-gel

filled emulsions. A 3D-matrix was visible also in cold-gel filled emulsions produced with CF, due to their ability to entrap water. All the parameters evaluated on emulsions were significantly affected ($p<0.001$) by the main experimental factors and their interactions. For the sake of brevity, only results about the type of emulsions are here commented and graphically showed (Fig. 1). In cold-filled gel emulsions, the lowest pH average values (3.97) and the highest average apparent viscosity levels (684 mPa·s) were determined. A similar apparent viscosity was obtained in hot-filled gel emulsions made with CP (627 mPa·s). The lowest average particle size (D90) was obtained in Pickering emulsions (77 µm) produced with CP in cold conditions. Consequently, Pickering emulsions showed the highest creaming stability (CS) after 14 days of storage (99.9%), while cold-filled gel emulsions had the lowest CS (98.8%) related to the highest oil droplet size (155 µm). As for stability index (SI), an interesting result was obtained using CP in the two different emulsions: SI was at maximum levels in hot-filled gel emulsions (99.5%), but at the minimum in Pickering emulsions (67%). These results can be related to the gelation capacity of chickpea proteins when heated (Karaca et al., 2011). With respect to the advanced emulsions, single emulsions made with lecithin showed in average a very low apparent viscosity (14.6 mPa s) and a low SI (52%). From the Pearson correlation matrix, a negative correlation was found between pH, apparent viscosity, and the particle dimensions, which had also a negative correlation with CS; this might be motivated by the fact that with the increasing of particle dimensions, instability phenomena (such as coalescence) are more frequent, promoting oiling off; a higher apparent viscosity, on the contrary, can promote a more compact structure in which the oil droplets are less prone to move and coalesce (McClements, 2015).

Figure 1 *MANOVA results for emulsion types (bars from left to right represent single, cold-filled gel, hot-filled gel, and Pickering emulsions; bars with different letters show significantly different results).*



3.2 Optimization with RSM technique

RSM results showed highly significant models for all the response variables ($p<0.0001$), except for pH ($p<0.05$), CS ($p<0.05$), and SI (not significant). Thus, the optimization was carried out with a desirability function that maximized the apparent viscosity and minimized the droplet size dimension (D90). In table 1 the optimized formulations that met all the criteria to develop a functional and stable delivery system are summarized.

Table 1 *Optimized formulations for the four types of studied emulsions.*

Emulsion type	Emulsifier type	Emulsifier (%)	Oil (%)
Single	Lecithin	2.5	58.49
Cold filled-gel	Citrus fibres	8.0	40.66
Hot filled-gel	Chickpea protein isolate	16.0	60.00
Pickering	Chickpea protein isolate	16.0	60.00

In table 2 results of technological parameters for the optimized emulsions are reported. pH of CF_cold_OPT resulted significantly lower, and this might have affected CS and SI, as well as the droplet size (Qi et al., 2021). The significant highest apparent viscosity of CP_hot_OPT is related to their ability to gelate and produce stable emulsions, with also small droplet size (Karaca et al., 2011).

Table 2 *Results of the optimized emulsion characterization.*

	Optimized emulsion samples			
Parameters	**Le_sing_OPT**	**CF_cold_OPT**	**CP_hot_OPT**	**CP_Pick_OPT**
pH	6.354 ± 0.205^{b}	3.906 ± 0.022^{a}	6.597 ± 0.013^{c}	6.635 ± 0.004^{c}
Apparent viscosity (mPa·s)	144 ± 12^{a}	1365 ± 46^{b}	2380 ± 123^{d}	1868 ± 57^{c}
D90 (µm)	19 ± 1^{a}	139 ± 6^{d}	83 ± 9^{c}	45 ± 8^{b}
CS t14d	100.0 ± 0.1^{b}	99.8 ± 0.1^{a}	100.0 ± 0.1^{b}	100.0 ± 0.1^{b}
SI t14d	88.7 ± 0.4^{a}	97.6 ± 0.1^{b}	100.0 ± 0.1^{c}	100.0 ± 0.1^{c}

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Developing a shelf-life predictive model for dry foods packed in biobased materials: the case study of coffee capsules

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The use of biobased packaging for dry foods is encountering some pitfalls, which are strictly related to the water vapor permeability. Furthermore, the barrier properties of these novel materials seems to be deeply affected by environmental storage conditions. The hierarchy of the physicochemical parameters influencing the quality of ground coffee packed in biobased materials was identified, and allowed to lay the foundation for a shelf-life prediction model.

Sviluppo di un modello di previsione di *shelf-life* per alimenti secchi confezionati con materiali bioplastici: il caso studio delle capsule di caffè

L'utilizzo di materiali di imballaggio per alimenti di tipo *biobased* presenta alcune criticità legate alla loro permeabilità al vapor d'acqua. Tale proprietà sembra essere influenzata dalle condizioni ambientali di conservazione. In questo lavoro è stata individuata la gerarchia dei parametri chimico-fisici che influenzano la qualità del caffè confezionato in capsule *biobased*. Questi risultati hanno consentito di porre le basi allo sviluppo di un modello predittivo di *shelf-life*.

Key words: ground coffee, shelf-life, dry foods, food packaging.

1. Introduction

The transition from petrol-based packaging materials to biobased ones represents a promising trend in the perspective to minimize environmental plastic pollution. However, biobased materials seem to exhibit a moisture sensitivity that is absent in conventional ones. This peculiar behavior is opening new and unexpected criticalities in the management of dry foods packed in biobased materials since these products might have a much shorter shelf-life than expected (Del Nobile et al., 2003). To face this emerging problem, the development of suitable shelf-life prediction models accounting for both the packaging dynamic performances towards environmental relative humidity (ERH) and the food moisture sensitivity are urgently required. The case study of roasted ground coffee packed in biobased capsules is here described.

2. Materials and Methods

Capsules made of polybutylene succinate (PBS), *i.e.*, bio-based, or polypropylene/ethylene-vinyl-alcohol/polypropylene (PP/EVOH/PP), *i.e.*, petrol-based, containing 7 g of freshly produced roasted coffee powder were prepared in an industrial packaging plant. Samples were stored in dark condition at 20 °C and 23, 54, 65 and 75% ERH. During storage, the coffee powder was retrieved from the capsules and analyzed for: *a)* water activity (a_w) at 25 °C by means of AQUALAB 4TE hygrometer; *b)* moisture (M) by gravimetric method according to AOAC (2000). At different storage times, capsules were picked up and used to obtain a coffee brew by using a domestic espresso coffee machine. After extraction, coffee brews were rapidly cooled down at 23 °C by means of a blast chiller and their pH was measured with a pH-meter (Basic20, Hach Lange Spain S.L.U. Riera Principal, Alella, Barcellona). The results reported are the average of at least 3 replicates.
Additionally, unpacked ground coffee powder was equilibrated at 20 °C and 11, 32, 43, 65, 75, 87, and 97% ERH by means of static, gravimetric method (Wagstaffe and Jowitt, 1990). M and a_w of coffee were analyzed and data were modelled using the Guggenheim-Anderson-De Boer (GAB) equation.

3. Results and Discussion

Figures 1a and *1b* show the effect of packaging material on moisture uptake and a_w evolution of coffee samples stored at 75% ERH and 20 °C. There is a noteworthy difference between the two chosen packaging solutions: samples stored in PBS capsules reached the equilibrium within few months for both M and a_w parameters. Similar trends were obtained at 23, 54 and 65% ERH (data not shown). Conversely, slight changes were detected in M uptake and a_w evolution for coffee packed in PP/EVOH/PP capsules. These results confirm the ineffectiveness of the bio-based packaging in protecting coffee from moisture uptake compared to the petrol-based packaging.

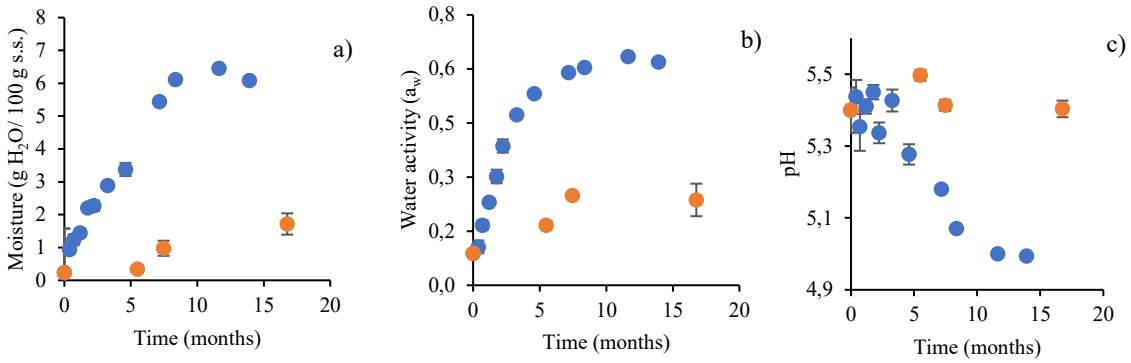

Figure 1 *Moisture uptake (a), a_w evolution (b), of ground coffee packed in PBS (•) and PP/EVOH/PP (•) capsules and stored at 20°C and 75% ERH. pH (c) of coffee brews obtained from coffee capsules having different storage time at 20°C and 75%ERH.*

Table 1 *Values of the GAB parameters obtained by fitting the experimental data.*

Parameter	Estimated values
m_0 (g H2O/100 g d.b.)	3.11
$a_{w,0}$	0.42
k	0.94
C	2.10
R^2	0.97

Observing *Figure 1a* and *1b*, coffee packed in PBS capsules reached the monolayer in 3 months while samples packed in PP/EVOH/PP ones did not reach this limit within the 15-month observation time. Since the water content of the coffee powder is expected to affect the pH of the coffee brew, capsules having different storage times at 75% ERH were extracted and the obtained brew was analyzed for the pH value (*Figure 1c*). The pH of coffee brews obtained from PBS capsules showed a biphasic behavior: after a 3 month-lag phase, a decrease in pH values was detected. By contrast, negligible pH changes were detected for coffee samples packed in PP/EVOH/PP. It is noteworthy that for PBS capsules, the pH lag phase corresponded to the period of time required for coffee moisture to approach the monolayer. Data relevant to coffee moisture uptake and pH evolution of the brew extracted from samples packed in PBS and stored at 20 °C and 23, 54 and 65% seem to confirm this hypothesis. In fact, in all cases, the pH decrease of the coffee brew started only when the moisture content of the coffee powder approached the monolayer (data not shown). The time needed to reach the monolayer was strongly dependent on the ERH (4 and 9 months at 54 and 65% ERH, respectively). Thus, the development of a shelf-life prediction model for coffee packed in PBS should take into careful consideration the effect of ERH on packaging moisture permeability. The latter not only strongly affects coffee moisture uptake but also triggers coffee staling upon monolayer approach. To this purpose the following function (Eq. 1) should be considered in developing a shelf-life model of coffee packed in PBS capsules:

$$SL = f\left(pH\left(a_w(P(ERH))\right)\right) \qquad (1)$$

where *SL* is the ground coffee shelf-life, *pH* is the critical indicator referred to the brew and related to the ground coffee quality, a_w is the physicochemical parameter that triggers coffee staling, and *P* is the water vapor permeability coefficient of the adopted packaging material. All these information will be used to predict the shelf-life of ground coffee packed in PBS capsules.

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Development of extracts and fermented officinal plants for food use endowed with sensory, antimicrobial and nutraceutical properties

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The first two activities of the PhD thesis project are described. Firstly, growth curves of fermenting bacteria were constructed obtaining the number of colonies per millilitre (CFU/ml). Secondly, *Calendula officinalis* aqueous extracts were fermented with strains of lactic acid bacteria for 24 hours, correlating colonies per millilitre (CFU/ml) and pH values.

Sviluppo di estratti e fermentati di piante officinali per uso alimentare con funzione sensoriale, antimicrobica e nutraceutica

Le prime due attività del progetto di tesi di dottorato sono descritte. In primo luogo, sono state costruite le curve di crescita dei batteri fermentanti, ottenendo il numero di colonie per millilitro (UFC/ml). In secondo luogo, gli estratti acquosi di *Calendula officinalis* sono stati fermentati con ceppi di batteri lattici per 24 ore, correlando il numero di colonie per millilitro (UFC/ml) e i valori di pH.

Key words: fermentation, lactic acid bacteria, plant extract, calendula.

1. Introduction

This poster reports the main results of the first two activities concerning:

1) construction of growth curves of the following fermenting bacteria: *Lactobacillus rhamnosus* GG, *Lactobacillus plantarum* 299V, *Pediococcus acidilactici* 12B and *Bacillus subtilis natto*;
2) *Calendula officinalis* aqueous extracts fermentation with *Lactobacillus plantarum* 299V and *Pediococcus acidilactici* 12B.

2. Materials and Methods

1) Fresh De Man, Rogosa and Sharpe (MRS) broth (peptone from casein 10 g/L; meat extract 8 g/L; yeast extract 4 g/L; D(+)-glucose 20 g/L; dipotassium hydrogen phosphate 2 g/L; Tween® 80 1 mL/L; di-ammonium hydrogen citrate 2 g/L; sodium acetate 5 g/L; magnesium sulfate 0.2 g/L; manganese sulfate 0.04 g/L.) was inoculated with 1% from -20 °C frozen stock bacterial cultures and incubated overnight at 37 °C; afterwards, new fresh MRS broth was inoculated with 0,01% from the overnight culture. This culture was plated by spread plate technique on MRS agar medium, every 2 hours from time zero to 10 hours, and then at 24 hours; plates were incubated overnight at 37 °C.
2) *Calendula officinalis* aqueous extract was inoculated with 10^5 CFU/ml of lactic acid bacteria (*Lactobacillus plantarum* 299V and *Pediococcus acidilactici* 12B, separately) and incubated overnight at 37 °C; the inoculated extract was plated at time zero and after 24 hours and its pH value was measured with pHmeter HI5521 (Hanna Instruments Inc., Woonsocket, United States).

3. Results and Discussion

3.1 Fermenting bacteria growth curves

Table 1 and Figure 1 show the kinetics of bacterial growth: for all four bacteria, the exponential phase starts approximately after 2 hours, but *L. rhamnosus* GG, *L. plantarum* 299V and *P. acidilactici* 12B reach the stationary phase approximately after 10-12 hours, while at that time *B. natto* begin to decrease the number

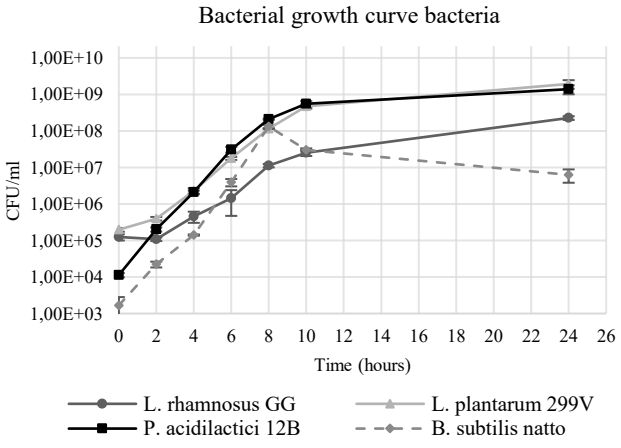

Figure 1. *Growth curves of L. rhamnosus GG, L. plantarum 299V, P. acidilactici 12B and B. natto plotted as CFU/ml on time.*

of viable cells, probably due to sporification process of this bacteria, that occurs once the stationary phase is achieved.

Table 1. *Number of CFU/ml of L. rhamnosus GG, L. plantarum 299V, P. acidilactici 12B and B. natto plated at different time points.*

Time (hours)	*L. rhamnosus* GG	*L. plantarum* 299V	*P. acidilactici* 12B	*B. natto*
0	$1{,}24*10^5 \pm 2{,}43*10^4$	$1{,}97*10^5 \pm 2{,}32*10^4$	$1{,}13*10^4 \pm 1{,}53*10^3$	$1{,}67*10^3 \pm 1{,}15*10^3$
2	$1{,}09*10^5 \pm 1{,}21*10^4$	$3{,}97*10^5 \pm 4{,}51*10^4$	$2{,}01*10^5 \pm 2{,}57*10^4$	$2{,}23*10^4 \pm 4{,}04*10^3$
4	$4{,}60*10^5 \pm 1{,}55*10^5$	$2{,}28*10^6 \pm 4{,}20*10^5$	$2{,}11*10^6 \pm 2{,}17*10^5$	$1{,}41*10^5 \pm 5{,}57*10^3$
6	$1{,}43*10^6 \pm 9{,}61*10^5$	$1{,}78*10^7 \pm 1{,}70*10^6$	$3{,}10*10^7 \pm 5{,}61*10^6$	$3{,}93*10^6 \pm 8{,}96*10^5$
8	$1{,}13*10^7 \pm 1{,}22*10^6$	$1{,}14*10^8 \pm 1{,}76*10^6$	$2{,}09*10^8 \pm 8{,}33*10^6$	$1{,}33*10^8 \pm 1{,}40*10^7$
10	$2{,}53*10^7 \pm 4{,}73*10^6$	$4{,}70*10^8 \pm 6{,}08*10^7$	$5{,}50*10^8 \pm 1{,}05*10^8$	$3{,}03*10^7 \pm 3{,}06*10^6$
24	$2{,}27*10^8 \pm 2{,}65*10^7$	$1{,}94*10^9 \pm 5{,}17*10^8$	$1{,}39*10^9 \pm 3{,}83*10^8$	$6{,}33*10^6 \pm 2{,}52*10^6$

All data are expressed as mean ± standard deviation.

3.2 *Calendula officinalis* aqueous extract fermentation

Table 2 and Table 3 report the number of CFU/ml in the inoculated calendula extracts plated at the moment of inoculum and after 24 hours of fermentation: *L. plantarum* 299V and *P. acidilactici* 12B were able to grow from about 10^5 to 10^8 CFU/ml.

The starting pH of calendula extracts was between 5,44 and 5,50 and both bacteria were able to lower it after 24 hours of fermentation, reaching values ranging from 3,74 to 4,03 (Table 2 and Table 3).

The fermented extracts appear more limpid, compared to the non-fermented ones, which present a higher number of particles in suspension (Figure 2), probably linked to consumption and/or transformation of plant material by bacterial metabolism.

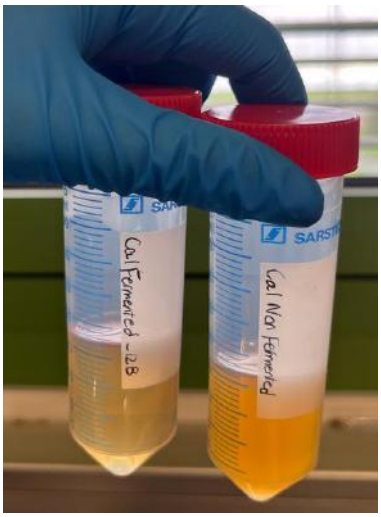

Figure 2. *Calendula extract non-fermented (right) and fermented (left) by P. acidilactici 12B.*

Table 2. *Number of CFU/ml of L. plantarum 299V in calendula extract, plated at time zero and after 24 hours of fermentation, with correspondent pH values.*

Time (hours)	CFU/ml	pH
0	$2{,}60*10^5 \pm 2{,}97*10^5$	5,50 ± 0,10
24	$2{,}90*10^8 \pm 1{,}07*10^8$	3,74 ± 0,058

All data are expressed as mean ± standard deviation.

Table 3. *Number of CFU/ml of P. acidilactici 12B in calendula extract, plated at time zero and after 24 hours of fermentation, with correspondent pH values.*

Time (hours)	CFU/ml	pH
0	$1{,}26*10^5 \pm 8{,}44*10^5$	5,44 ± 0,025
24	$5{,}87*10^8 \pm 7{,}29*10^8$	4,03 ± 0,04

All data are expressed as mean ± standard deviation.

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Insight into *in-vitro* digestibility of leavened baked goods

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A Type I sourdough was prepared using traditional methods. It was fermented for 72 hours at 30°C with *Lactiplantibacillus plantarum* CR1, *Furfurilactobacillus rossiae* CR5, and *Saccharomyces cerevisiae* E10. The mature sourdough was used to make sourdough bread. A control bread was made with 1.5% (w/w) baker's yeast and fermented for 2 hours at 30°C. The sourdoughs were analyzed for pH, total titratable acidity (TTA), and organic acids. An *in vitro* simulation of the upper gastrointestinal tract was used to digest both bread samples and monitor nutrient mapping. This protocol (by PRODIGEST) includes specific enzymes and processing parameters that mimic in vivo digestion optimally.

Approfondimenti riguardo la digeribilità *in vitro* dei prodotti da forno lievitati

È stato preparato un lievito madre di tipo I utilizzando un protocollo tradizionale inoculando *Lactiplantibacillus plantarum* CR1, *Furfurilactobacillus rossiae* CR5 and *Saccharomyces cerevisiae* E10. L'impasto ha fermentato per 72 ore in tutto. L'impasto maturo è stato usato per produrre il campione di pane a lunga fermentazione, che è stato poi confrontato con un pane preparato con 1,5% di lievito birra. Gli impasti sono stati analizzati in base ai valori di pH, acidità totale titolabile (TTA) e acidi organici e sottoposti ad una simulazione *in vitro* del processo digestivo. Il protocollo imita il processo di digestione in vivo, includendo specifici enzimi digestivi e considerando diversi parametri.

Key words: Sourdough, baked goods, digestibility.

1. Introduction

In accordance with the papers of Rizzello *et al*. (2019) and Da Ros *et al*. (2021), this poster shows the main results of the first two activities concerning:

(A1) Preparation of experimental sourdough and baker's yeast breads. Sourdough fermentation and characterization in terms of pH values, total titratable acidity (TTA), and organic acids and bread making;

(A2) *In vitro* pre-digestion process of bread samples. The static *in vitro* simulation of human gastrointestinal digestion consists of 3 phases: the oral phase, the gastric phase and the small intestine phase;

(A3) TWINSHIME® (UGent/ProDigest) experiment. Configuration consisting of three consecutive bioreactors simulating stomach and small intestine together, and proximal (PC) and descending (DC) colon

2. Materials and Methods

Type I sourdough was made and propagated using *Lactiplantibacillus plantarum* CR1, *Furfurilactobacillus rossiae* CR5 and *Saccharomyces cerevisiae* E10. The inoculum corresponded to ca. 5×10^7 and ca. 5×10^6 cfu/g for lactic acid bacteria and yeasts, respectively. The dough, with a Dough Yield of 160, was incubated at 30 ◦C for 16 h. Further the first fermentation, four back slopping steps were carried out, mixing 20% of the previously fermented dough with flour and water and incubating at 30 ◦C for 8 h. A final refreshment was carried out at 30°C for 24 h with the same parameters of inoculum and DY. During each refreshment, sourdough aliquots were analysed in terms of pH, TTA and organic acids (lactic and acetic acids) by High Performance Liquid Chromatography (HPLC) with an UV detector operating at 210 nm. Baker's yeast and sourdough bread samples were manufactured according to Rizzello et al. (2019). The *in vitro* simulation of human gastrointestinal digestion consisted of 3 phases: the oral phase, the gastric phase and the small intestine phase. The oral pre-digestion included food dilution with simulated salivary fluid and the exposure (2 min) to salivary amylase. Then, the oral bolus was diluted with simulated gastric fluid and gastric enzymes (pepsin and gastric lipase) and incubation lasting at 37 ˚C for 2 h, using a pH gradient from 6.0 to 2.0. Finally, the gastric chyme was diluted with simulated intestinal fluid, bile salts and pancreatic enzymes and incubation at pH 7 was extended for further 3 h at 37 ˚C under static dialysis with a membrane of 14 kDa. Samples were further analyzed using the SHIME® (the Simulator of the Human Intestinal Microbial Ecosystem) to evaluate the effect of breads on the gut microbiota functionality. The set-up was a TWINSHIME® (UGent/ProDigest) configuration consisting of three consecutive bioreactors simulating stomach and small intestine together, and proximal (PC) and descending (DC) colon. Before starting, all the bioreactor that mimic the colon tracts were inoculated with a representative healthy fecal sample from the same donor. Samples have been collected from the SHIME® bioreactors at T_3 fecal material collected from

ascending colon tract, at T_4 from traversal colon tract and at T_5 from descending colon tract.

3. Results and Discussion

3.1 Biochemical characteristics of sourdough

After the last fermentation, the mature sourdough reached the value of pH of 3.59 ± 0.005. Resulting pH for each fermentation was time dependent. The longer the duration of fermentation, the lower was the value of pH. Values of TTA resulted to be in line with the organic acids produced.
The concentration of acetic acid was 20.5 ±0.3 mM after each fermentation steps. In comparison, the content of lactic acid was higher after 16 and 24 h of fermentation (64.2 mM and 53.5 mM, respectively). The fermentation quotient ranged from 3.5 and 4.8.

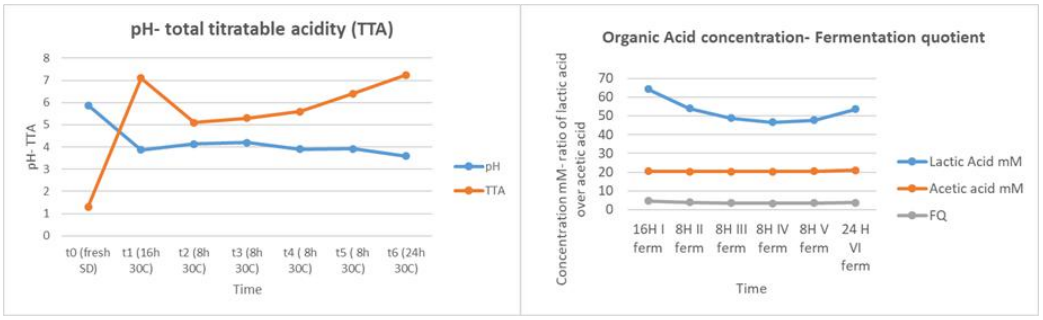

Figure 1. *Physico- chemical and biochemical characteristics of the sourdough. pH, total treatable acidity (TTA) (mL of NaOH /pH 8.3), organic acid concentration and fermentation quotient of the sourdough at each back slopping steps.*

3.2 Sampling

The *in vitro* static simulation of the upper gastrointestinal tract and colon tract allowed for the acquisition of all the necessary samples for subsequent analysis, enabling the mapping of factors that influence the digestibility of sourdough breads and their impact on the human gut microbiome and metabolome. Specifically, bolus samples were collected following the oral phase of *in vitro* digestion, chyme samples following the gastric phase and chyle and permeate samples following the small intestine phase. The kilo, from the last step of the *in vitro* simulation of the upper gastrointestinal tract, was tested in the SHIME® in order to continue with macronutrients mapping even into the large intestine simulation. The SHIME® experiment proceeded with a stabilization and control phase of the microbial community in the bioreactors, followed by a one-week treatment period where the gut microbiota was nourished with a media that simulated daily meals supplemented with digested sourdough bread. During this phase, faecal lumen samples were collected for subsequent biochemical analysis.

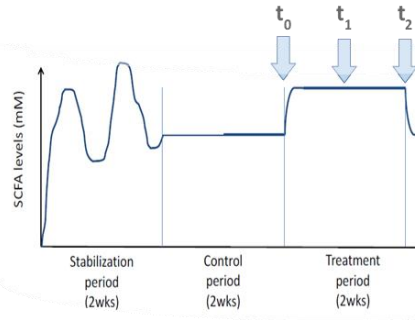

Figure 2. *Timing* SHIME® *experiment*

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ProDigest Gastrointestinal Expertise, Dialysis for Twinshime® installation and operating manual.

Study of optical, mechanical and permeability properties of sustainable packaging solutions for cured meat products

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This Ph.D. project aims to study the functional properties of sustainable packaging solutions specifically designed for cured meat products. In this work, optical, mechanical, and permeability properties of sustainable tray systems were investigated and compared with multilayer plastic materials suitable for cured meat products.

Studio di proprietà ottiche, meccaniche e di permeabilità di packaging sostenibili per prodotti carnei affettati

Lo scopo di questo dottorato è quello di studiare materiali di imballaggio sostenibili per prodotti carnei affettati. Pertanto, in questo lavoro le proprietà ottiche, meccaniche e di permeabilità di materiali di confezionamento sostenibili sono state studiate e comparate con materiali plastici multistrato utilizzati per il confezionamento di prodotti carnei affettati.

Key words: Food packaging, cured meat, barrier properties, optical properties, sustainability.

1. Introduction

Common packaging for cured meat products relies on plastic multi-material trays and lids. However, these solutions are not environmentally sustainable as they derive from non-renewable sources, moreover, they are not biodegradable or compostable and the recycling process is still an open issue due to challenges with layer division (Horodytska et al., 2018). In accordance with the Ph.D. project, this poster reports the main results of the following activity:

(A1). Study of the optical, mechanical and permeability properties of a PET mono-material, and bio-based packaging compared with conventional multilayer plastic solution for cured meat products.

2. Materials and Methods

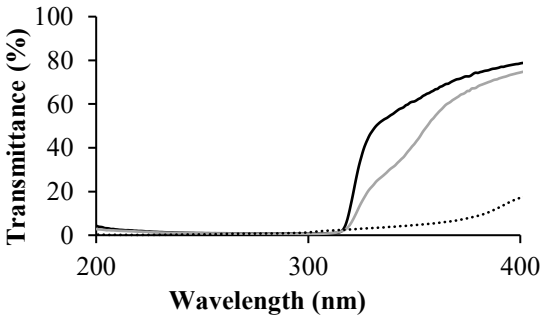
A standard multilayer structure, made of APET-PE/EVOH/PE (T-STD_{250}), were compared with alternative solutions: a mono-PET (PET/r-PET/PET) (T-PET), and a compostable biopolymer (T-Bio). Standard PET-PE-EVOH lid (L-EVOH) was compared with SiO_x-coated PET (L-PET_{SiOx}) and a biopolymer film (L-Bio). Optical properties (transparency and haze) were evaluated through a UV-Vis spectrophotometer (Lambda 650, PerkinElmer, Waltham, MA, USA). Mechanical properties (Young's modulus (YM), elongation-at-break (EAB), and tensile strength (TS)) were measured using a dynamometer (Zwick Roell, Ulm, Germany) in machine (MD) and transverse direction (TD). Permeability properties of carbon dioxide (*CO_2TR)*, oxygen (*O_2TR)*, and water vapor barrier (*WVTR)* were determined with a permeability analyser (Extrasolution Srl, Capannori, Italy). The statistical analyses (one-way ANOVA, Tukey's post-hoc, Dunnett's T3) were carried out using the SPSS 27 software (SAS, Cary, NC).

3. Results and Discussion

3.1 Optical properties

Tray materials displayed a decrease in transmission within the range of 200 – 400 nm (Figure 1), thus indicating good protection against UV light (Domínguez et al., 2019).

Figure 1. *UV-Vis transmission spectra from 200 nm to 800 nm of tray packaging films. Legend: T-STD_{250} (black solid line), T-PET (grey solid line), T-Bio (black dotted line).*



T-STD_{250} and the T-PET films exhibited the highest transmittance values within the visible region (84% < T_{550} < 86%), which indicates good transparency. These results can be attributed to the presence of amorphous PET, the main component of these tray films, characterized by a high clarity (Nisticò, 2020). However, the T-Bio film exhibited very low transmittance values at 550 nm (29%), due to the intrinsic opacity of the films. Among lid films, the transparency which is more important since it allows consumers to view the food products, ranged between 87 to 89% (data not shown).

3.2 Mechanical properties

The plastic standard multilayer film exhibited YM value of ~ 1900 MPa in both machine and transverse directions. Nevertheless, the T-PET film showed significantly ($p < 0.05$) higher values of YM (2289 and 2250 MPa for TD and MD, respectively) as compared to the standard multilayer film, caused by the absence of PE layers which display good toughness. As expected, the T-Bio was characterized by the greatest value of YM, as well as by the lowest EAB value, which is attributed to the high brittleness and low plasticity of biobased polyesters (De Beukelaer et al., 2022).
Regarding the lid films (Table 1), L-EVOH exhibited good mechanical properties, thus showing good ductility, with a percentage of EAB ranging between 39% and 47%. Interestingly, the L-PET_{SiOx} film showed the highest value of YM, together with acceptable extensibility (29% and 39% in TD and MD, respectively). The high YM of L-PET_{SiOx} is related to the presence of the rigid silicon oxide layer ($YM_{metal} \approx 80$ GPa) (Howells et al., 2008). The L-Bio carried a higher YM and significantly lower EAB in MD (7 %) as compared to the standard material ($p < 0.05$), due to the PLA layer which provides brittleness and rigidity (Pietrosanto et al., 2020).

Table 1. *Values of mechanical parameters for the lid materials.*

Lid sample	E (MPa)		EAB (%)		TS (MPa)	
	TD	MD	TD	MD	TD	MD
L-EVOH	1343 ± 152^{a}	1356 ± 43^{A}	39.9 ± 7.0^{b}	47.5 ± 7.2^{B}	37.8 ± 2.0^{a}	37.8 ± 1.1^{A}
L-PET_{SiOx}	4575 ± 488^{c}	4698 ± 144^{C}	29.5 ± 7.6^{a}	40.0 ± 7.1^{B}	133.8 ± 17.9^{c}	135.3 ± 8.8^{C}
L-Bio	2817 ± 347^{b}	2998 ± 337^{B}	$^{*}33.6 \pm 5.6^{ab}$	$^{*}7.2 \pm 3.9^{A}$	63.8 ± 2.3^{b}	65.5 ± 2.6^{B}

Significant differences ($p < 0.05$) among materials when evaluated in TD (lowercase letters) or MD (uppercase letters), respectively. * Indicates a significant difference ($p < 0.05$) between MD and TD for the same material

3.3 Barrier properties

Regarding tray films, the CO_2 barrier properties of T-Bio (0.53 $cm^3\ m^{-2}\ day^{-1}$) exhibited a significantly higher value ($p < 0.05$) compared to the standard multilayer film (1.53 $cm^3\ m^{-2}\ day^{-1}$). It is interesting to note that water vapour transmission rates were analogous ($p > 0.05$) between T-PET, and T-Bio.
Regarding lid films, L-EVOH and L-PET_{SiOx} showed comparable CO_2TR and O_2TR values ($p > 0.05$), suggesting that both the applied barrier film, respectively EVOH and SiO_x, were accountable for the limited transfer of carbon dioxide and oxygen through the lid films (Korte et al., 2023). L-Bio exhibited statistical differences ($p < 0.05$) in CO_2TR compared to L-EVOH and L-PET_{SiOx}. Finally, the comparison between samples concerning the WVTR parameters revealed that materials performed fine with similar values. In these results, the L-EVOH film lid was characterized by a significantly higher value (4.97 g $m^{-2}\ day^{-1}$) compared to the other ones ($p < 0.05$).
The results obtained in this study showed the potential of alternative solutions to replace multilayer plastic materials designed for cured meat products, which can be exploited in future packaging development.

4. Acknowledgment

This work was carried out in collaboration with the University of Milan together with Dr. Daniele Carullo and Prof. Stefano Farris.

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Identification of aggregate phenolic metabotypes after an Oral (Poly)phenol Challenge Test (OPCT) and their association to the cardiometabolic health status of 300 subjects

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This PhD thesis project aims at identifying aggregate metabolic phenotypes (metabotypes) for the main dietary (poly)phenols, evaluating the determinants of inter-individual variation leading to different metabolic profiles and building predictive algorithms for a faster identification of metabotypes. Three hundred volunteers underwent an oral (poly)phenol challenge test (OPCT) in the form of 3 (poly)phenol-rich tablets, also providing personal information on their health status as well as biological samples to assess urinary phenolic excretion, gut microbiota composition and genetic polymorphisms. This intervention study will allow to find associations between cardiometabolic health and (poly)phenol metabolism.

Identificazione dei metabotipi fenolici aggregati a seguito di una challenge nutrizionale per lo studio del metabolismo dei (poli)fenoli e la loro associazione allo stato di salute cardiometabolico di 300 soggetti

Questo progetto di tesi di dottorato mira ad identificare i fenotipi metabolici aggregati (metabotipi) per i principali (poli)fenoli della dieta, valutare i fattori della variabilità inter-individuale che portano ai diversi profili metabolici e costruire algoritmi predittivi per una più rapida identificazione dei metabotipi. Trecento volontari si sono sottoposti ad una challenge nutrizionale (OPCT) consumando 3 compresse ricche di (poli)fenoli, fornendo informazioni personali sul proprio stato di salute nonché campioni biologici per valutare l'escrezione fenolica urinaria, la composizione del microbiota intestinale e la genotipizzazione. Questo studio di intervento permetterà di trovare un'associazione tra la salute cardiometabolica e il metabolismo dei (poli)fenoli.

Key words: metabotypes, dietary challenge, (poly)phenols, cardiometabolic health.

1. Introduction

Increasing evidence suggests that modest long-term intakes of (poly)phenols can reduce the risk of chronic diseases, especially cardiovascular diseases and type 2 diabetes (Rodriguez-Mateos et al., 2014). Nevertheless, the role of (poly)phenols in cardio-metabolic protection has not been consistently demonstrated yet (Gibney et al., 2019). The inter-individual variability plays an important role in the physiological response, mainly influenced by differences in the absorption, distribution, metabolism, and excretion (ADME) of (poly)phenols (Gibney et al., 2019), along with other factors, including genetic background, gut microbiota, sex, age, ethnicity, lifestyle (diet, smoking, and physical activity), (patho)physiological status and medication (Gibney et al., 2019; Cassidy & Minihane, 2017). After ingestion, (poly)phenols reach the colon, where they undergo modifications by the gut microbiota, being converted to smaller catabolites, principally as conjugated phase II metabolites, which can act as mediators of diet-induced effects on health (Del Rio et al., 2013). The inter-individual differences in gut microbial composition and functionality can lead to quantitative and qualitative differences in the production of specific metabolites, influencing the bioactivity of (poly)phenols in the host (Manach et al., 2017). The different catabolite production patterns may be related to metabolic phenotypes (the so called metabotypes).
This acute human intervention study is directed to understand the association between aggregate metabolic phenotypes for the main dietary (poly)phenols and the factors determining their formation.

2. Materials and Methods

An intervention study was carried out on 300 volunteers who met specific inclusion and exclusion criteria. Recruited subjects were healthy adults (18-74 y) with a BMI ranging between 18.5 and 35.0 kg/m^2, free from cardiometabolic diseases and impairments mostly related to the gastro-intestinal tract, renal and liver functionality. During Visit 1, after signing the informed consent, they were asked to provide dietary and lifestyle information and to undergo anthropometric measurements. Clinical data and biological samples (blood, urine, and faeces) were delivered at Visit 2 when subjects underwent a standardised oral (poly)phenol challenge test (OPCT) consisting in an acute supplementation of up to 15 classes of dietary (poly)phenols in the form of 3 tablets. Urine samples

collected during the following 24-h were analysed through UPLC-IMS-HRMS to assess the individual urinary excretion of phenolic metabolites, allowing clustering according to aggregate metabotypes. Blood samples were analysed to determine common cardiometabolic health biomarkers (total cholesterol, HDL-cholesterol, triglycerides, glucose, insulin, etc.) and for whole-genome genotyping focused on genetic polymorphisms (SNPs). Faeces were subjected to microbial profiling to determine gut microbiota composition at species level. Cardiometabolic risk scores were also assessed.

3. Results and Discussion

Up to 298 volunteers finished the study. Preliminary analyses showed that the cohort was made up of 57% of women, having an average age of 40.7 y (SD ± 16.3); regarding anthropometric measures, 73% of the sample had a normal weight, 22% was overweight and 5% obese. The mean values of the clinical data concerning cardiometabolic health ranged within the reference values. A preliminary targeted approach was performed on 187 subjects for the identification of more than 100 (poly)phenol metabolites and to allow population clustering according to different metabotypes. The preliminary results showed two main metabotypes defined by differences related likely to the gut microbiota composition; indeed, among all the phenolic metabolites identified, the most discriminating ones were those of colonic origin (e.g., enterolactones, phenyl-γ-valerolactones) (Figure 1 and 2). Final data on metabotypes and cardiometabolic risk will be presented in the poster. Individuals metabolise dietary (poly)phenols in different ways and the interlink among different families of (poly)phenols has been described. Further analyses are ongoing to provide a deeper knowledge on inter-individual variability determinants involved in metabotype formation and its relation to the cardiometabolic health status.

Figure 1. *Partial least squares-discriminant analysis (PLS-DA) highlighting two main metabotypes.*

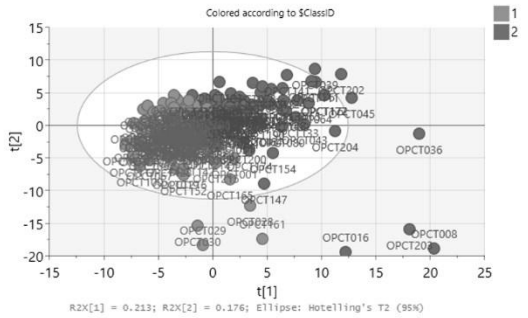
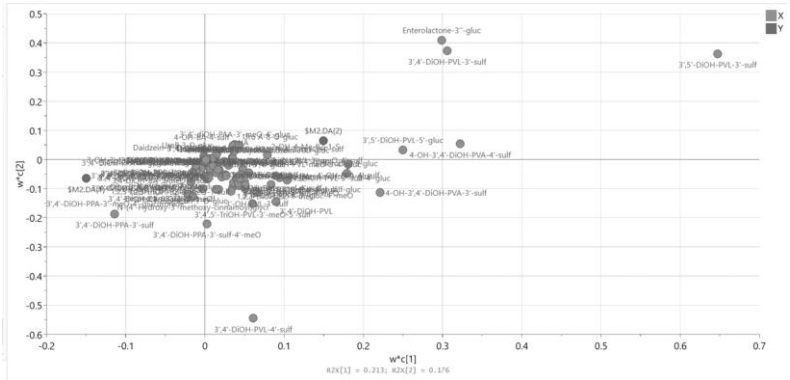


Figure 2. *Partial least squares-discriminant analysis (PLS-DA) showing the urinary excretion of colonic phenolic metabolites (phenyl-γ- valerolactones and enterolactones).*



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Sensory and technological improvement of hemp seed flour. Strategies for food applications.

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The first two activities of the PhD project are described: 1) characterization of two by-products of the hemp oil production process, namely hemp seed cake flour (HSCF) and hemp seed protein concentrate (HSPC); 2) use of the two by-products as fortifying ingredients in gluten-free baked goods.

Miglioramento delle proprietà sensoriali e tecnologiche della farina di canapa. Strategie per il settore alimentare.

Sono descritte le prime due attività del progetto di tesi di dottorato: 1) caratterizzazione della farina ottenuta dal pannello di canapa (HSCF) e del concentrato proteico di semi di canapa (HSPC), due sottoprodotti del processo di estrazione meccanica a freddo dell'olio di canapa; 2) utilizzo dei suddetti sottoprodotti come ingredienti alimentari per fortificare prodotti da forno senza glutine.

Keywords: hemp seed cake flour; hemp seed protein concentrate; hemp seed oil by-products; flour techno-functionality; volatile profile; gluten-free bakery products.

1. Introduction

In accordance with the PhD thesis project, this poster reports the main results of the first two activities concerning: (A1) the assessment of the nutritional and techno-functional properties, and of the volatile profile of two by-products of the hemp oil production process, namely hemp seed cake flour (HSCF) and hemp seed protein concentrate (HSPC); (A2) the development of gluten-free muffins fortified with different percentage (15%, 20%, 30%) of HSCF or HSPC and the evaluation of their nutritional, technological and functional quality.

2. Materials and Methods

2.1 Characterization of HSCF and HSPC

The HSCF and HSPC were analyzed for their proximate composition. Moisture, ash, protein (N × 5.7), and fiber content were determined according to the official methods (Horwitz and Latimer, 2005). The total fat content was determined on hexane Soxhlet extracts. Carbohydrate content was calculated by difference. Folin-Ciocalteu assay was used for the total phenolic content (TPC) determination. Bulk density (BD), water holding capacity (WHC), oil absorption capacity (OAC), and swelling index (SW) of both HSCF and HSPC were assessed according to Okaka & Potter (1977). The volatile profile of the two by-products has been investigated by HS-SPME-GC-MS; for the HS-SPME extraction, 5 g of each sample was suspended in 15 mL of saturated sodium chloride solution in a 40 mL vial. Extraction was performed at 35 °C exposing a DVB/CAR/PDMS fiber, 50/30 µm film thickness (Supelco, Bellefonte, PA, USA), to the headspace of the sample for 30 min. The sample was maintained under continuous magnetic stirring and, before extraction, thermally balanced for 30 min. The extracted analytes were directly desorbed into the injector port of the GC/MS held at 260 °C. The GC-Ms analyses were performed as previously reported by Condurso et al. (2020).

2.2. Development of fortified Gluten-free muffins and assessment of their nutritional, technological and sensory quality.

Gluten-free muffins were prepared according to the method described by Shevkani *et al*. (2015) using rice flour, sunflowers oil, sugar, egg, milk, and baking powder. HSCF or HSPC were used as partial substitutes for rice flour in three different percentages, i.e. 15%, 20%, and 30%. Three muffin samples fortified with HSCF, three fortified with HSPC, and one control sample (CM) were produced on a laboratory scale and their technological quality has been assessed through texture profile analysis (TPA) (Martínez-Cervera *et al*., 2015), measurement of color based on the CIELab color system, and determination of cooking properties (moisture, volume, height, diameter, backing loss). CATA, hedonic, and acceptability tests, performed by a panel of 80 untrained judges, were used for the sensory quality evaluation.

3. Results and Discussion

3.1 Characterization of HSCF and HSPC

The HSCF showed a statistically higher content of fat and fiber and a lower content of protein, carbs, and TPC (Table 1), than HSPC. HSCF had a higher OAC due to its higher amount of insoluble fiber, and lower WHC and SW values as a consequence of its lower protein and carb contents. The higher OAC of HSCF makes it more suitable than HSPC to be used in bakery products where oil is an important ingredient, whereas its lower value of WHC will result in a lower level of available moisture in the baked goods where the flour is used affecting their textural properties and accelerating the process of crumb firming.

Table 1 *Gross composition, total phenolic content (TPC) and techno-functional properties of hemp seed by-products.*

	Moisture†	Fat†	Protein†	Carbs†	Ash†	Fiber†	TPC§	BD$	WHC†	OAC†	SW#
HSCF	10.14±1.1	9.6±1.0	26.1±2.1	5.1±0.3	0.05±0.0	46.3±4.1	7.21±0.8	0.60±0.00	204±2.8	56.0±0.0	1.39±0.02
HSPC	9.91±0.9	7.4±0.8	40.3±3.5	10.7±0.9	0.05±0.0	24.2±2.2	13.11±1.2	0.59±0.05	233±17.6	48.5±0.1	1.63±0.29
	ns	*	**	**	ns	**	*	ns	*	*	*

*†= (g/100g) and; §= mg gallic acid /100g; $=g/mL; #=adimensional; ns=not statistically significant; *= P<0,05; **= P<0,01*

The volatile profile of HSCF and HSPC was constituted mainly by alcohols, aldehydes, furanic compounds, and terpenoids (Figure 1); among volatiles compounds, 3-methyl-1-butanol (14.36 %), 1-hexanol (12.19%), hexanal (10.63%), 3-methyl-butanal (8.73%), and 2-pentyl-furan (8.33%) prevailed in HSCF, whereas 3-methyl-1-butanol (10.68%), hexanal (10.57%), 1-hexanol (8.17%), acetic acid (7.38%), and (Z)-2-heptenal (5.6%) were the main constituents of the HSPC volatile fraction. Most of the identified volatile compounds are plant secondary metabolites resulting from the different biosynthetic pathways occurring in plant tissue. Others, such as 2-alkylfurans, originate from oxidation of unsaturated fatty acid denoting unsuitable packaging and storage conditions of the flour.

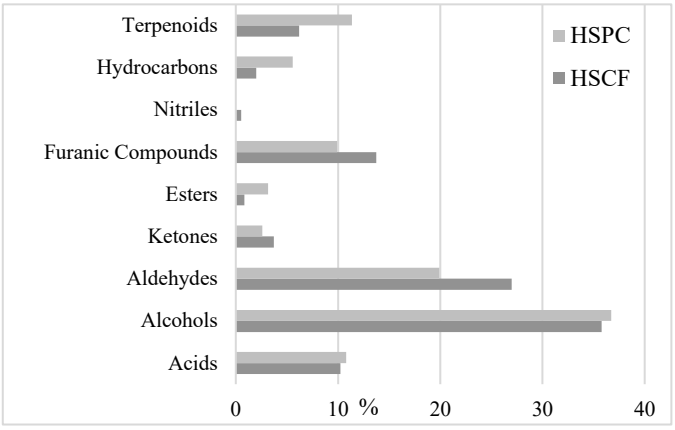

Figure 1 *Volatile composition as class of substances (%) hemp seed by-products.*

3.2. Development of fortified Gluten-free muffins and assessment of their nutritional, technological and sensory quality.

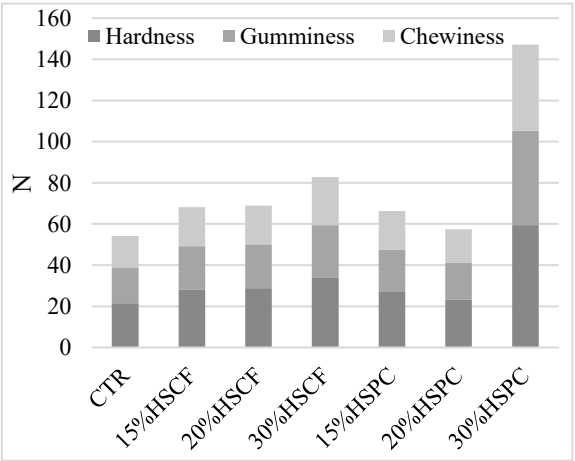

Figure 2 *TPA results of control and fortified gluten-free muffins.*

The moisture of the gluten-free muffins was higher than the control samples, especially for muffin samples fortified with HSPC as expected due to the higher WHC of HSPC than HSCF. The incorporation of HSCF and HSPC did not affect the baking loss, but improved the physical parameters (muffin volume, specific volume and height) of muffins except for sample with 30% of HSPC. Regarding crust color, fortified muffins showed lower values for L* a* b* than the control sample: the higher was the percentage of fortifying flours, the greater the decrease of crust color parameters. Also crumb L* and b* values decreased with the increasing percentage of the HSCF or HSPC, whereas the a* values increased, denoting a tendency to crumb browning. The hardness of HSCF muffins was always higher than the control, whereas in the case of HSPC muffins, the hardness increased at a 30% addition level; a similar trend was observed for the muffin gumminess and chewiness for both by-products (Figure 2). The consumer's acceptability of fortified samples was similar to the control samples, except for muffins fortified with 30% HSPC which was the least appreciated by the consumers. The results suggest that both HSCF and HSPC are suitable as fortifying ingredients for gluten-free muffins.

Exploiting wine lees to improve nutritional features of biscuits

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Wine lees flour was used in the production process of biscuits to enrich them in fibres, proteins, and phenolic compounds, thus improving their nutritional value. Control biscuits were compared with biscuits obtained by replacing refined flour with 10 or 20% wine lees flour. The results of the analytical determinations showed that the experimental biscuits were richer in fibres, proteins, ashes, and phenolic compounds, resulting in an increase in antioxidant activity. Sensory analysis, however, showed that astringency, bitterness, and acidity increased in proportion to the amount of wine lees flour used.

Valorizzazione delle fecce di vino per migliorare le caratteristiche nutrizionali dei frollini

La farina di fecce di vino è stata utilizzata nel processo produttivo di frollini per arricchirli in fibre, proteine e composti fenolici, al fine di migliorarne il valore nutrizionale. Frollini controllo sono stati confrontati con quelli ottenuti sostituendo la farina raffinata con la farina di fecce di vino nella misura del 10 e 20%. I risultati delle determinazioni analitiche hanno dimostrato che i frollini sperimentali erano maggiormente ricchi in fibre, proteine, ceneri e composti fenolici, con conseguente incremento dell'attività antiossidante. L'analisi sensoriale, invece, ha evidenziato che astringenza, amaro e acidità si incrementavano proporzionalmente al quantitativo di farina di fecce utilizzato.

Key words: Oenological by-products, wine lees flour, biscuits, dietary fiber, antioxidant activity, polyphenols.

1. Introduction

Oenological by-products are becoming increasingly important in the context of food applications; in fact, scientific evidence suggests their properties, functionality, and benefits that promote human health when administered in food formulations (Sharma et al., 2015). In particular, wine lees are known to be rich in dietary fiber and phenolic substances with antioxidant properties (Sharma et al., 2022). Therefore, this work aims to enhance the value of wine lees by using them as a source of bioactive molecules for the fortification of baked goods. The choice of fortifying the biscuits was dictated by the need to improve their nutritional composition because in most cases conventional biscuits are made using refined flours, sugars and fats that give high caloric power and fibres poverty (Devi et al., 2016). This poster presents the main results of the activities regarding treatment of wine lees, the formulation of biscuits enriched with wine lees flour and their characterization.

2. Materials and Methods

2.1 Wine lees flour

Wine lees were freeze-dried, reaching moisture values of 3% or less. Flour production was carried out by mixing and subsequent passing in a hammer mill (Dietz-motoren KG). Then, the sieving by vibrating (Giuliani, Turin, Italy) at 300 rpm for 40 min was conducted.

2.2 Biscuits formulation and characterization

The classic recipe for this type of product was used as a control sample (F0) and in experimental biscuits wine lees flour (WLF) replaced 10% (F10) and 20% (F20) of wheat flour. The recipe was as follows: wheat flour (F0, 250 g; F10, 225 g; F20, 200 g), wine lees flour (F0, 0 g; F10, 25 g; F20, 50 g), semi-skimmed milk (80 g), olive oil (70 g), sugar (70 g), and ammonium bicarbonate (3 g). Then, after kneading, rolling, and forming, the biscuits were baked in a ventilated electric oven for 16 minutes at 160 ºC. The moisture content was measured using a thermobalance (MAC 110/NP). The proteins, ashes, lipids, and total dietary fibres (TDF) contents were determined using the methods 979.0, 923.03, 945.38 F and 985.29, respectively (AOAC, 2006). Carbohydrates were determined by subtracting the values of proteins, ashes, lipids, and moisture from 100. The polyphenols extraction from biscuits was carried out according to the protocol described by Leal et al. (2020) with some modifications. Extracts were utilized for determination of antioxidant activity (AA), by ABTS and DPPH tests, and total phenol content (TPC) by Folin-Ciocalteu according to Noviello et al. (2022). All analysis mentioned were carried out in triplicate. A panel group of 10 people at the University of Bari Aldo Moro was trained to conduct the sensory analysis of the biscuits. Visual and tactile analyses was evaluated through biscuits colour (0= yellow; 9= light brown/violet) and friability (0= very hard; 9= very crumbly), respectively. The gustatory attributes subjected to evaluation were sweetness, saltiness, acidity, bitterness, astringency, and off taste intensity according to the following score (0= unperceived; 9= very intense). Finally, the material attributes perceived upon tasting was assessed by evaluating the following parameters: hardness (0= soft; 9 =hard), dryness (0= humid; very dry), and granularity (0= no perceived particle; 9= many particles of various sizes). The hardness (N) of biscuits was evaluated also using a texture analyser (Z1.0 TN, Zwick Gmbh & Co., Ulm, Germany). The method applied was

the 3-point bending test using 1 KN load cell, the distance between the distance bars was 4 cm with a probe speed of 5 mm/min. Six replicated were made for each sample.

2.3 Statistical analysis

Statistical processing was carried out using Minitab 17 (Minitab Inc., State College, PA, USA) subjecting the data to analysis of ANOVA variance and Tukey test for multiple comparisons.

3. Results and Discussion

Table 1 shows proximate composition, antioxidant activity, total phenol content and hardness of biscuits. A significant increase in proteins and ashes in fortified biscuits was observed compared to the control one due to the addition of wine lees rich in these components (data not shown). The proteins increase was in line with the results obtained by Sharma et al. (2022) in yogurt enriched with wine lees. In addition, TDF was significantly higher in F10 and F20 than in F0, allowing the nutrition claim "fibre source" to be obtained due to fibre content greater than 3 g/100 g in F10 and "high fibre content" due to the presence of TDF exceeding 6 g/100 g in F20. As a result, higher humidity was also observed in the experimental biscuits than in the control, probably due to the greater fiber content brought by wine lees and having affinity towards the components of water allow it to be retained inside the matrix (Maner et al., 2017). Consequently, carbohydrates significantly decrease in the experimental biscuits. The results obtained from the characterization of biscuits showed a dose-dependent increase of TPC and AA with a higher rate of replacement of the wine lees flour in biscuits formulation. These results agree with the literature where the increase of phenols and the antioxidant activity observed in ice cream (Sharma et al., 2015) are linked to the addition of wine lees. Phenols and anthocyanins analysis for HPLC showed that in experimental biscuits, as well as in WLF, ellagic acid, malvidin 3-glucoside and malvidin 3-acetyl-glucoside were the most abundant. Finally lower hardness was found in experimental biscuits (F10, F20) than in the control sample (F0), probably due to the dilution of gluten caused by WLF which does not contain gluten.

Table 1. *Proximate composition, antioxidant activity, total phenol content and texture profile analysis of biscuits.*

Sample	F0	F10	F20
Moisture (g/100 g)	4.80 ± 0.03 ^{b}	9.05 ± 0.06 ^{a}	9.23 ± 0.20 ^{a}
Proteins (g/100 g)	8.46 ± 0.00 ^{b}	9.94 ± 0.15 ^{a}	9.57 ± 0.32 ^{a}
Lipids (g/100 g)	16.50 ± 0.15 ^{b}	17.82 ± 0.29 ^{a}	17.25 ± 0.04 ^{ab}
Total dietary fiber (g/100 g)	2.67 ± 0.09 ^{c}	4.70 ± 0.12 ^{b}	8.04 ± 0.33 ^{a}
Ashes (g/100 g)	0.44 ± 0.01 ^{c}	1.53 ± 0.04 ^{b}	2.68 ± 0.01 ^{a}
Carbohydrates (g/100 g)	67.12 ± 0.02 ^{a}	56.97 ± 0.02 ^{b}	53.23 ± 0.91 ^{c}
ABTS (µmol TE/g)	1.16 ± 0.09 ^{c}	2.43 ± 0.08 ^{b}	4.27 ± 0.08 ^{a}
DPPH (µmol TE/g)	0.47 ± 0.00 ^{c}	2.63 ± 0.05 ^{b}	4.77 ± 0.13 ^{a}
TPC (mg GAE/g)	0.25 ± 0.03 ^{c}	0.70 ± 0.05 ^{b}	1.45 ± 0.01 ^{a}
Hardness (N)	53.28 ± 2.03 ^{a}	25.70 ± 0.26 ^{b}	22.93 ± 0.30 ^{c}

F0, control biscuits without wine lees; F10, F20 biscuits with 10% and 20% wine lees flour. Data are represented as means ± SD of three lots and different letters in the same row mean a significant difference at $p < 0.05$.

The sensory properties of experimental biscuits were strongly influenced by the addition of wine lees. Panellists reported an increase in violet colour proportional to the addition of wine lees. The WLF addition led to a friability increase directly proportional to the increase in the percentage of substitution. The perception of acidity, bitterness, astringency, and off-taste intensity significantly increased with higher levels of WLF added. As expected, the acidic taste found in experimental biscuits is due to the acids contained in WLF. The bitterness and astringency sensation can be attributed to the presence of polyphenols in WLF flour due to the interaction that occurs between polyphenols and saliva (Davidov-Pardo et al., 2012). Finally, texture attributes as hardness, dryness and granularity were influenced by WLF addition. Specially, the lower hardness of the experimental biscuits (F10, F20) compared to the control sample (F0), agrees with the instrumental results (hardness). Similarly, the dryness of F10 and F20 was less than control; this could be associated with the increased humidity of the experimental biscuits. Although richer in fiber and phenolic compounds, panellists rated F20 with a higher perception of acidity, bitterness, astringency, and taste than F10.

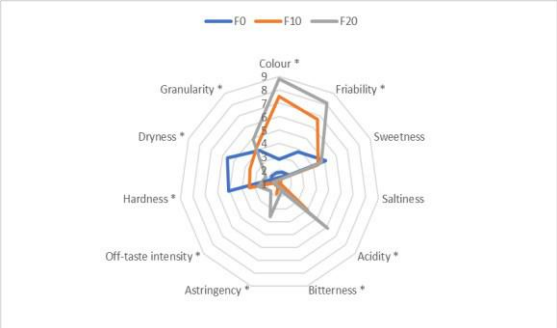

Figure 1. *Results of the sensory analysis of biscuits; data are represented as means ± SD of ten panellists.*

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Exploring the Biotechnological Potential of *Starmerella bacillaris* beyond winemaking

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In the wine industry, where *Saccharomyces cerevisiae* yeast derivatives are commonly used to enhance wine quality attributes, there is still a lack of knowledge regarding the utilization of non-*Saccharomyces* strains derived products. In the first part of this PhD program, different strains of *Starmerella bacillaris,* a non-*Saccharomyces* yeast naturally present on the grape skin surface, were grown in grape must-like conditions. After cell harvesting, cell wall and cytoplasm were separated by means of mechanical lysis. Carbohydrates and protein content of these two fractions were then investigated in comparison with those obtained from *Saccharomyces cerevisiae,* grown in same conditions, spotting key differences between these species, in a framework of evaluating the capabilities of *Starmerella bacillaris* lees recycling for derivatives manufacturing.

Starmerella bacillaris come risorsa biotecnologica oltre la vinificazione

Nell'industria enologica moderna, i derivati di lievito ottenuti da *Saccharomyces cerivisae* sono utilizzati per migliorare gli attributi qualitativi del vino. Tuttavia, poco conosciuto risulta ancora l'impiego di derivati ottenuti da specie non-*Saccharomyces*. Nella prima parte di questo programma di dottorato sono stati propagati in condizioni fermentative diversi ceppi di *Starmerella bacillaris*. Dopo aver collezionato la biomassa, le pareti cellulari e il contenuto citoplasmatico sono stati separati a mezzo di lisi meccanica. La composizione in carboidrati e proteine di queste due frazioni sono state determinate e messe a confronto con quelle ottenute da *Saccharomyces cerevisiae,* propagato nelle stesse condizioni. È stato quindi possibile individuare le principali caratteristiche di *Starmerella bacillaris* per un prossimo sviluppo di derivati di lievito applicabili in enologia

Key words: *Starmerella bacillaris*, non-saccharomyces yeast, yeast cell wall, yeast protein extracts

1. Introduction

This PhD project funded by PON resources and carried on with the close partnership and economic support of Groupe SOFRALAB (Magenta – France) is focused on yeast derivatives applications in winemaking. Products obtained by yeasts are applied to improve qualitative attributes of wine mimicking in a controlled manner the spontaneous ageing on lees process, which has been carried on from decades by wine producers simply maintaining the wine in contact with the leftover of the alcoholic fermentation (Ángeles Pozo-Bayón *et al.,* 2009). Although the use of yeast derivatives in winemaking is currently limited to those derived from *Saccharomyces cerevisiae* strains (OIV OENO 576A-2017), there is a growing interest in non-*Saccharomyces* species with the aim of discovering new biotechnological tools for the winemaking process. Additionally, the demand for circular economy practices encourages the reuse of by-products, such as wine fermentation lees in winemaking. For this reason, the work conducted so far focused on the characterisation of lees of *Starmerella bacillaris,* a wine yeast that has shown high physiological variability and desirable technological properties (Raymond Eder and Rosa 2021), in two cellular fractions that finds application in yeast derivatives sector: cell walls and yeast extracts.

2. Materials and methods

Biomass growth and cell harvesting

A set of 6 strains of *Starmerella bacillaris* (***SB***) and 6 strains of *Saccharomyces cerevisiae* (***SC***) were inoculated at 10^6 cells/mL in MS300 synthetic must (Bely, Sablayrolles, and Barre 1990) at 200 g/L of glucose and fructose in 1:1 ratio and pH 3,2. Fermentations were performed at 25 °C under agitation for 40 hours. Cell concentration during the growth was monitored with a Cyflow cytofluorimeter (Sysmex Partec, Goerlitz, Germany).

Cell lysis

Cell breakage protocol was defined based on works of Avramia & Amariei (2022), with glass beads (425 – 600 µm of diameter from Sigma, St. Louis, MO, USA). After recovery of the liquid fraction, the lysed cells suspension was centrifuged at 5200 g for 30 minutes and supernatant, accounting for soluble cytoplasmic matters, was collected and lyophilized as whole extract (WE); the pellet containing the insoluble yeast cell walls (YCW) was washed for three times and lyophilized.

Table 1. *Sugar composition of yeast cell wall (YCW). Results are expressed as grams per 100 grams of cell wall dry weight. Different letters stand for significative differences at p<0,001*

Specie	*Mannose*	*Glucosamine*	*Glucose*
Saccharomyces cerevisiae	$23,38^{a} \pm 0,52$	$0,82^{b} \pm 0,13$	$29,98\%^{a} \pm 0,90$
Starmerella bacillaris	$20,64^{b} \pm 0,39$	$2,26^{a} \pm 0,12$	$19,89^{b} \pm 0,67$

Table 2. *Sugar composition of whole extract (WE). Results are expressed as grams per 100 grams of whole extract dry weight. Protein content is measured in the same way as BSA equivalent. Different letters stand for significative differences at*

Specie	*Mannose*	*Glucosamine*	*Glucose*	*Proteins*
Saccharomyces cerevisiae	$5,22^{b} \pm 0,41$	$0,82^{b} \pm 0,10$	$34,28^{a} \pm 2,66$	$19,85^{b} \pm 1,59$
Starmerella bacillaris	$7,12^{a} \pm 0,30$	$1,70^{a} \pm 0,07$	$16,36^{b} \pm 1,98$	$30,27^{a} \pm 1,30$

Acid hydrolysis and carbohydrates analysis

To hydrolyse carbohydrates composing the two fractions, freeze-dried material (WE and YCW) was treated according to Dallies et al. (1998) with H_2SO_4 in vacuum sealed tubes. Analysis of monomers liberated from acid hydrolysis was performed upon derivatization according to Wang et al. (2020). Protein concentration of WE were determined with Bradford assay kit (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as calibration standard. Protein visualisation was obtained through SDS-PAGE adapted from Laemmli (1970).

3. Results and discussion

Yeast cell wall (YCW)

As depicted in Table 1, *SB* resulted to have a cell wall richer in glucosamine and poorer in mannose and glucose, resulting in a global lower sugar concentration. As these three sugars are obtained during the acid hydrolysis starting respectively from chitin, mannoproteins and glucan, it is possible to infer some characteristic of *SB* composition. Firstly, the lower relative carbohydrates content comparing to *SC*, mainly driven by glucan content, suggests a cell wall for SB characterized by the higher presence of other components like proteins or lipids. Secondly, the chitin and its metabolism seem to play a much important role in the cell wall of this specie. It is possible to hypothesize a relevant role in must chitinase binding capabilities for cell walls of *SB* as this kind of activity has been reported in Chardonnay and model wine as dependent on chitin cellular concentration (Ndlovu, Divol, and Bauer 2018).

Whole extract (WE)

The cytoplasmic material of *SB* (Table 2) follows the same trend, except for mannose content. The most noticeable difference in WE composition between species regards however the protein content, where *SB* marks an average +52% in respect to *SC*. Proteins are not only quantitatively different, but on SDS-PAGE those coming from *SB* exhibit a wider molecular size distribution in the range 200 – 31 KDa, this might be of interest to obtain protein-based derivatives with oenological applications. The lower amount of WE glucose in *SB* might be impacted by a lower capability of storing reserve carbohydrates, like glucose and trehalose, in comparison to *SC*.

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Exploring barriers and enablers for the environmental sustainability communication "Zero-waste supply chain": insights from the Italian fruit & vegetables supply chain trough the LOWINFOOD project

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The first two activities of the PhD dissertation projects are described. Firstly, the fresh fruit and vegetable value chain was analysed to assess the factors that affect the adoption process of innovations at different entry points in the chain. For each entry point, a set of innovations has been reviewed and correlated with the ones available in the LOWINFOOD project. Secondly, the "Zero-waste supply chain" framework was theoretically defined. The exploration of the factors affecting the adoption process of innovation in the upper part of the value chain was made with both qualitative and quantitative methodologies.

Esplorare fattori abilitanti e barriere riferite alla comunicazione di sostenibilità ambientale "Filiera Zero-rifiuti": approfondimenti per la filiera italiana di Frutta & Verdura attraverso il progetto LOWINFOOD

Le prime due attività del progetto di tesi di dottorato sono riportate. La catena del valore dell'ortofrutta fresca è stata analizzata per valutare i fattori che influenzano il processo di adozione delle innovazioni nelle diverse fasi della catena. Per ogni fase, un insieme di innovazioni è stato studiato e correlato con approcci manageriali innovativi. In seguito, è stato definito il quadro teorico del framework "Zero-waste supply chain". L'esplorazione dei fattori che influenzano il processo di adozione dell'innovazione nelle fasi iniziali della catena del valore è stata effettuata con metodologie sia qualitative che quantitative.

Key words: Fruit & Vegetables, Innovation, Sustainability, Waste, Prevention, Recycling

1. Introduction

In accordance with the PhD thesis project previously described and the Gantt diagram provided in the Dissertation Project, this poster reports the main results of the first two activities concerning:
A1) Mapping problems and solutions in the F&V supply chain: To give an overview on the State of Art on the available innovations and actions in the F&V value chain to reduce food waste and losses.
A2) Driver and barriers to the introduction of sustainability innovation inside an organization: This is the core part of the project when will be defined a framework for the "*Zero-Waste supply chain*" assessment and the direct measurement of drivers and barriers is conducted on the upstream part of the fresh F&V value chain.

2. Materials and Methods

A literature review (Mengist et al., 2020) was conducted to map the available innovations in the FV value chain that aim to improve the management of surplus products and food waste. This approach allowed to identify the innovations that have already been adopted by different stakeholders and discuss the theoretical drivers and barriers to their adoption. The "Zero waste supply chain" framework is defined taking into consideration the available literature on the topic, the outcomes of A1) and the work done in the LOWINFOOD project. Three case studies have been selected as part of the framework. This includes the Production phase, Distribution & Retailer phase and Consumers. The direct assessment of the adoption factors of the Production phase of the value chain was performed on the upstream stakeholders of the value chain using both a qualitative and a quantitative methodology. The qualitative approach is based on the Q methodology by Stephenson (Brown, 1993). The outcome of this technique allows the methodical collection, examination, and comparison of stakeholders' opinions (Mandolesi, 2015). The quantitative assessment is performed through the development of a duration analysis model (1).

$$Pd = f(H, Ec, T, I) \quad (1)$$

Where:
Pd Probability of adopting the technology
H Vector of variables describing organization's characteristics and preferences
Ec Vector of variables describing economic factors
T Vector of variables describing technology characteristics
I Vector of variables describing institutional factors

Pd is the probability of adopting an innovation which is function of vector of variables. In particular, the vector of the PO's characteristics and preferences (H) was built also considering the outcomes of the quantitative analysis.

3. Results and Discussion

3.1 Mapping innovations in the fresh fruit and vegetables value chain

Using the review methodology in environmental sciences developed by Mengist (Mengist et al., 2020), the PICOC (Population, Intervention, Comparison, Outcomes, Context) framework was defined to review the available innovations in F&V value chains to prevent and valorise food waste. 553 paper was studied to create theoretical categories of innovations and sort them by entry points in the value chain. The outcomes shows that novel action with positive impact on actors sustainability is related to new decision support systems, the development of digital platforms, and new managerial approaches. The "Zero waste supply chain" framework involves identifying stakeholders and defining impact indicators. Scientific literature provides references together with the activities of the LOWINFOOD project. The framework evaluates the effectiveness of innovations in reducing waste along the supply chain, considering economic, environmental, and social aspects. By applying it to stakeholders, the impact of implemented innovations is measured.

3.2 Exploring barriers and enablers for innovations in the fresh F&V value chain

3.2.1 Qualitative Analysis

The application of the Q methodology was made to a sample of 14 Producer's Organizations (OPs), Association of OPs and supply – chain to explore the different social perspective of the upstream stakeholders about the thematic of innovations for the management of fresh F&V surplus and waste. The analysis revealed the preferences towards innovations for the two factors extracted for both the cluster of OPs and AOPs compared with supply chain experts cluster. The first cluster emphasizes a preference for organizational innovation towards the optimization of food surplus and food waste, e.g., redistribution to people in need. The second cluster shows opposite preferences; Factor 1 is essentially related to preferences towards market innovation oriented towards the prevention of food waste, while Factor 2 attitudes' are more focused on the recycling and recovery phases of food waste.

3.2.2 Quantitative Analysis

The Duration Analysis is used to observe, in the period 2012–2023, the effect due to the POs and APOs digital platform adoption on firm assets and gross sales and to evaluate the CAP-OCM market measures efficacy improvement linked to that innovation. The results show in detail how different factors, such as economic, institutional, and environmental, affected the adoption process over time. The research studies the time (T) between the moment the innovation is available and the adoption of organizations. In duration analysis studies the transition probability to a new state is defined as the Hazard, and is the conditional probability that an organization who does not belong to the platform selects to be part of in the short period of time *dt* after *t*. The Hazard function (2) is defined as:

$$H(t) = \lim_{dt \to 0} \frac{Pr\,(t \le T < t+dt \mid T \ge t)}{dt} = \lim_{dt \to 0} \frac{F(t+dt)-F(t)}{dt(1-F(t)} = \frac{f(t)}{S(t)} \quad (2)$$

The model developed also provides the possibility of calculating the probability that an organization with certain characteristics will take part in the platform after *t* years from its creation. S(t) is the survival function, indicating the probability that an organization will adopt the innovation in a time *t.* The Survival function S(t) indicates the probability that the random variable *T* is larger than *t* (3).

$$S(t) = exp\left\{-\int_0^t H(s)ds\right\} = 1 - F(t) = Pr(T \ge t) \quad (3)$$

The proportional hazard model is estimated by maximum likelihood for discrete time periods of one year, a Weibull baseline hazard function and a distribution of the unobserved heterogeneity following a Gamma distribution using the method proposed by Jenkins.

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Sustainable innovation to improve the quality of meat products in the era of the Green Deal

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This document describes two activities that were completed as part of the PhD project. The antioxidant potential of 120 Lactic Acid Bacteria (LAB) strains belonging to *Lactiplantibacillus* and *Lacticaseibacillus* spp. was first investigated. Then, a screening to evaluate Nitric Oxide Synthetase (NOS) activity among 50 strains of *Staphylococcus* spp. and LAB strains was also carried out.

Innovazione sostenibile per migliorare la qualità dei prodotti carnei nell'era del *Green Deal*

Nel presente documento vengono descritte due delle attività svolte nell'abito del progetto di dottorato. In primo luogo, è stato investigato il potenziale antiossidante di 120 ceppi di batteri lattici appartenenti a *Lactiplantibacillus* e *Lacticaseibacillus* spp.. Successivamente è iniziato uno *screening* per indagare la capacità di ceppi di *Staphylococcus xylosus* e di batteri lattici di produrre Ossido Nitrico Sintetasi (NOS).

Key words: antioxidant activity, lactic acid bacteria, Nitric Oxide Synthase, *Staphylococcus xylosus*.

1. Introduction

In recent years, there has been a significant increase in the demand for products with clean labels, that is, products with ingredients that are considered natural and healthy. Several authors have demonstrated that LAB have antioxidant capacity and thus could be used to replace synthetic antioxidants in meat products. Furthermore, some LAB and *Staphylococcus* spp. strains have been shown to produce NO from L-arginine via NOS. The activities presented in this work concern the screening of 120 LAB strains for antioxidant potential (A1) and the screening of LAB and *S. xylosus* strains for NOS activity (A2).

2. Materials and Methods

2.1. Antioxidant activity (A1)

120 strains of LAB belonging to the species *Lacticaseibacullus casei, Lcb. paracasei, Lcb. rhamnosus* and *Lactiplantibacillus plantarum*, isolated from different reservoirs such as dairy products, wine and wine cellars, bread dough, faeces, human body, and coffee, were tested for their antioxidant potential after adaptation under both anaerobic condition and after activation of aerobic metabolism, as reported by Zotta *et al.* (2014). The cells were grown for 18 h at 30 °C, then they were centrifuged (6000 xg, 10 min), the pellet was resuspended in PBS (Sigma-Aldrich, Milan, Italy) and after standardization of their concentration, an inoculum of 10^6 CFU/mL was performed in MRS broth and supplemented M17 broth. This step was repeated twice and finally after centrigugation the supernatants and the washed cells were analysed using the DPPH method as described by Cao *et al.* (2019) and Yu *et al.* (2020), while two commercial kits, ABTS Assay Kit and Ferric Reducing Antioxidant Power (FRAP) Assay kit (Bioquochem, Asturias, Spain) were adopted following the manufacturer instructions for the ABTS and FRAP assay, respectively.

2.2. NOS enzymatic activity in *S. xylosus* and Lactic Acid Bacteria (A2)

Nitric Oxide Synthase activity of 50 strains of *S. xylosus* were investigated using a commercial kit Nitric Oxide Synthase (NOS) Activity Assay Kit (Colorimetric) (Sigma-Aldrich, Milan, Italy), following the protocol reported by the manufacturer.
Finally, 100 µL of each strain suspension was standardized at 10^7 CFU/mL and inoculated in MRS broth (LAB) and Luria Bertani (LB) broth (*S. xylosus*) (Oxoid, Milan, Italy) supplemented with 20 mg/mL myoglobin (Sigma-Aldrich, Milan, Italy) and 50 mM L-arginine (Sigma-Aldrich, Milan, Italy). After incubation for 18 h at 30 °C, the suspensions were centrifuged, the supernatant was collected and subjected to UV-Vis analysis with a Tecan Sunrise microplate reader (Tecan Italia Srl, Cernusco sul Naviglio, Italy) and the absorption spectrum between 450 and 700 nm was obtained (Ras *et al.*, 2018; Luo *et al.*, 2020; Xu *et al.*, 2023).

3. Results and Discussion

3.1. Antioxidant activity

One of the main causes of meat quality depletion after microbial alteration is oxidation, which cause quality depletion, and the accumulation of toxic compounds, which can lead to the onset of non-transmissible chronic diseases (Carocho *et al.*, 2018). For these reasons, the antioxidant activity of the different strains was evaluated. From DPPH and ABTS analysis all the stains seemed to present antioxidant activity, however, FRAP analysis didn't confirm these data. There was no correlation between antioxidant activity expressed by strains and their growth pattern, both under aerobic or anaerobic metabolism, but the antioxidant potential resulted to be strain-specific. The only exception was in the case of the cellular pellet grown under aerobic conditions, of which results showed lower average antioxidant activity than the other conditions. It was also discovered that the cells of aerobically grown strains had activity equal to, or lower than, the control consisting of ascorbic acid (1.5 ppm), whereas the other treatments have at least 20% of the strains with higher activity than the control. These findings are very promising, but they must be confirmed *in vivo* before to substitute the synthetic antioxidants in meat products with the use of this strains as bioprotective cultures.

3.2. NOS enzymatic activity

Nitrate and nitrite salts are commonly used to maintain the bright red colour of meat, but the residual content of nitrites reduces the ability of red blood cells to bind and transport oxygen through the body and contributes to the formation of nitrosamines (EFSA ANS Panel, 2017). Several studies have shown that bacteria such as *Staphylococcus* spp. and LAB can produce NO from L-arginine via the NOS enzyme (Yarullina *et al.*, 2011; Ras *et al.*, 2017; Xu *et al.*, 2023). The preliminary screening of 50 out of 100 strains belonging to the DI4A revealed that none of them showed NOS activity, as all of the tested strains had enzymatic activity levels lower than the detection limit of the used method (5 pmol/min/µg). As a result, additional research is being conducted to identify a producer strain by evaluating the bacteria's ability to develop colour in a broth culture supplemented with Met-Myoglobin and arginine. Same results were obtained for LAB, since none of the tested strains demonstrated the ability to produce NO from arginine. Indeed, no absorbance peaks were identified in the absorption spectra of the tested strains at 581 nm, the typical wavelength of NO-myoglobin. Thus, further research is required.

4. Conclusions

A central theme in implementing the quality of meat products is the search for natural alternatives to synthetic antioxidants. To this purpose, a screening was carried out to assess the antioxidant activity of LAB strains in order to replace the compounds currently in use. It was discovered that the strains tested have good *in vitro* potential, though this needs to be confirmed *in vivo* as well. Furthermore, a screening was performed to determine whether strains of *S. xylosus* and LAB can produce NO from L-arginine via the NOS enzyme in order to reduce the concentration of nitrites in meat products. However, none of the tested strains demonstrated this ability, so additional research is being conducted.

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Exploring the use of plant-based flours and fermentation for the production of a legume-avocado based vegan cream cheese

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This PhD project aims to use flours derived from various plant sources to make a vegan cream cheese in which oil source has been replaced with fresh avocado. After the selection of the flours, a fermentation step also took place, and the product was evaluated, concerning nutritional and textural parameters, compared to the control non-fermented one.

Esplorazione dell'uso di farine vegetali e della fermentazione per la produzione di un formaggio cremoso vegano a base di legumi e avocado

Questo progetto mira a utilizzare farine derivate da diverse fonti vegetali per produrre un formaggio cremoso vegano in cui l'olio è stato sostituito da avocado fresco. Dopo la selezione delle farine è stata effettuata anche una fase di fermentazione. Il prodotto finale fermentato è stato valutato in termini di parametri nutrizionali e di texture, e confrontato con il corrispondente prodotto non fermentato (controllo).

1. Introduction

Cheese is considered a highly nutritional food, with cow's milk being an essential component of the human diet. However, sustainability is becoming an important consideration for industry and thus, there is a growing need for the production of plant-based prototypes (Grossmann & McClements, 2021). Along with the nutritional features, fermentation allows us to optimize the flavor, appearance, and overall quality and safety of these products. This project is divided into three different steps. The first one is dealing with the selection of the flour and flour-water ratio, the second one with the selection of the combination of starter cultures, and the last one with the evaluation of final cheese attributes.

2. Materials and Methods

As a first step, chickpea, red lentils, quinoa, and fava flours were tested in different percentages, and cheeses were evaluated for their textural and sensory attributes. For the selection of the best-performing flours and the percentage that is going to be used in the recipe, a commercial stracchino vegan cream cheese was used as a control. Vegan cheeses were prepared based on a patent proposed by Ferawati et al. (2021) with some modifications. As a second step, different species of lactic acid bacteria (LAB), such as *Lactococcus lactis, Lactiplantibacillus pentosus, Lacticaseibacillus paracasei, Apilactobacillus kunkeei,* and *Lacticaseibacillus rhamnosus* together with a commercial starter, were evaluated for their ability to ferment the different cheese matrices. Unfermented samples including the same ingredients and following the same process were used as controls. Based on the results, different clusters were created, and from each of these, a representative strain was selected to create the best combination of starters for each cheese matrix. As a last step, vegan cheeses made from the selected flours were fermented with the selected combination of LAB starters. Texture, color, and sensory characteristics were evaluated, along with further characterization concerning peptides, free amino acids, and free fatty acid formation, volatile organic compounds, dietary fibers, polyphenols, starch hydrolysis, and antinutritional factors.

3. Results and Discussion

After the screening of the four flours, we decided to proceed with the red lentil and chickpea flours since they gave us a more similar textural profile with respect to our control sample (commercial cheese). Additionally, the two above-mentioned flours were the most preferable ones among all, according to their sensory attributes.
Different starter cultures were evaluated by measuring the pH, organic acid production, cell density, viscosity and spreadability together with the evaluation of sensory attributes. Based on the results from the screening, control sample (without fermentation) and sample of spontaneous fermentation were grouped in the same cluster while strains were grouped in three different clusters. *Lactiplantibacillus pentosus*, *Lacticaseibacillus paracasei*, *Lacticaseibacillus rhamnosus* and *Apilactobacillus kunkeei, Lactiplantibacillus pentosus, Lacticaseibacillus*

paracasei were selected as representative strains from each cluster, for chickpea and red lentil, respectively (Figure 1).

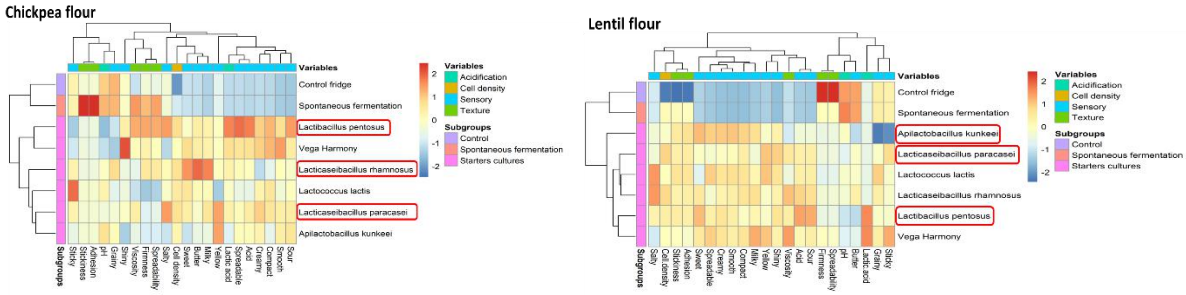

Figure 1. *Heatmaps of the chickpea and red lentil vegan cream cheeses showing the acidification, cell density, sensory and texture results after fermentation with different strains of lactic acid bacteria.*

Final plant-based cheese samples were fermented with different combinations selected for chickpea and red lentil separately. The evaluation of the nutritional and textural attributes of the final cheeses led to different profiles between fermented and nonfermented ones. Concerning the antinutritional factors such as phytic acid, raffinose, and saponins, both chickpea and red lentil cheeses showed lower values (15.1± 0.1, 26.5± 0.1, 34.0± 1.0 mg/g of dry matter for chickpea and 6.2± 0.1, 15.5± 0.1, 17.9± 1.0 mg/g of dry matter for red lentil, respectively) in the fermented samples compared to the unfermented ones (15.7± 0.1, 30.6± 0.1, 42.2± 1.0 mg/g of dry matter for chickpea and 6.8± 0.1, 21.8± 0.1, 26.6± 1.0 mg/g of dry matter for red lentil, respectively). Additionally, compared to the control, fermented samples had less content of starch indicating the impact of the starter cultures on starch hydrolysis. With respect to dietary fibers, once again there was a statistical difference ($P < 0.05$) between the fermented and not fermented ones, with the last ones having higher values. Phenolic compounds and free fatty acids were found in both cheeses, proving the nutritional impact of the presence of avocado. Finally, the results of the *in vitro* protein digestibility and the total free amino acids, together with all the above cases, confirmed the positive effect of lactic acid fermentation in both samples (Filannino, P., et al., 2020).

4. Upcoming work

A non-destructive method will be utilized with an AR-G2 controlled stress rheometer to analyze changes in texture during the six hours of fermentation by measuring G', G", and tan(delta). After six hours, samples will be subjected to a creep-recovery test to determine their viscoelastic qualities. Turbiscan will be used to evaluate emulsifying activity and stability as a final step.

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Industry 4.0 in the agri-food sector: innovative sensors and smart logistics to support the sustainability of the supply chain

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The aim of this PhD research project is to find solutions that can objectively measure the quality of high value-added productions. This will be achieved through the creation of simplified portable devices that exploit vis/NIR spectroscopy technology. This technology is already widely used in these sectors but requires complex and expensive instrumentation. The implementation of new smart sensors could lead to improvements in production, increased awareness of supply chain operators, more effective management of resources and ultimately, a reduction in waste. The latter in particular is one of the final objectives of the entire agri-food chain.

Keywords: vis/NIR spectroscopy, sensors, industry 4.0, non-destructive, food quality.

Industria 4.0 nel settore agroalimentare: sensori innovativi e logistica smart a supporto della sostenibilità di filiera

L'obiettivo di questo progetto di ricerca di dottorato è trovare soluzioni che possano misurare in modo oggettivo la qualità di produzioni ad alto valore aggiunto. Ciò verrà realizzato attraverso la creazione di dispositivi portatili semplificati che sfruttano la tecnologia della spettroscopia vis/NIR. Questa tecnologia è già ampiamente utilizzata in questi settori, ma richiede strumentazione complessa e costosa. L'implementazione di nuovi sensori smart potrebbe portare a miglioramenti nella produzione, a un aumento della consapevolezza degli operatori di filiera, a una gestione più efficace delle risorse e, infine, a una riduzione degli sprechi. Quest'ultimo in particolare è uno degli obiettivi finali di tutta la filiera agroalimentare.

1. Introduction

There is currently a significant shift occurring in measurement technologies within the agri-food sector. There is growing interest in replacing traditional laboratory-based analytical techniques with rapid, non-destructive, and environmentally sustainable methods (Nicolai et al., 2007). One effective approach is the utilization of visible and Near Infrared (vis/NIR) spectroscopy and imaging techniques. These methods have been extensively researched and utilized in the agri-food sector for their numerous advantages, including non-destructive sampling, fast results, and the ability to conduct checks throughout production processes. However, the instruments required for these techniques are often expensive, complex to operate and unsuitable for settings outside specialized laboratories.
To address this, researchers are focusing on developing low-cost prototypes with comparable characteristics to reliable laboratory instruments, albeit with slightly lower performance (Tugnolo et al., 2021). These devices could be deployed in larger quantities along the supply chain, allowing precise quality controls due to their affordability. By conducting meticulous quality checks, the aim is to deliver products of the highest quality while addressing customer interests in healthiness and sustainability. Chemometrics is critical for proper data interpretation, predictive modelling, and variable selection, essential for simplifying devices.
This PhD program aims to offer a potential solution to the requirements of high value-added supply chains by implementing and applying a simplified spectroscopic device that uses only a limited number of wavelengths (dos Santos Costa et al., 2019). These devices will be able to facilitate punctual and real-time analysis, guaranteeing the production of high-quality finished products while minimizing waste.

2. Materials and Methods

This PhD thesis project has been divided into several activities. Initially it was applied a portable device, originally built for the analysis of small matrices such as grapes and olives, on larger matrices such as figs. The aim, in addition to the creation of predictive models, was to identify the required hardware modifications on the first version of the prototype.
Subsequently, the experiments focused on matrices that can be considered a cross between a solid matrix and a liquid matrix (must) or grapes at winery consignment. These grape samples (Vitis Vinifera L.) variety "Ancellotta" were analysed with a bench/process spectrophotometer with a lab scale experimental setup reproducible at the winery. Samples were analysed both in the laboratory and at the winery with the same instrument and the same sampling methods and predictive models were created for the measurement of some qualitative parameters and the polyphenol content.
The experiments then moved on to the creation of a simplified prototype for the analysis of liquid matrices, with the intention of applying it to liquids such as wine to identify adulterations but also to denser samples such as for

example musts (also in this case to measure qualitative parameters and polyphenol content), to be able to try to carry out the same analyses conducted with the bench instrument with this new simplified version. The pre-prototype was built using a 3D printer and, inside the case built through additive manufacturing, it was assembled low-cost sensors (Hamamatsu Photonics) with a module in the visible wavelengths range and a module in NIR range. The experiments of the prototype for the liquid matrices started analysing fruit juices to verify the best experimental setup to proceed with the analysis of wines and to verify the effective functioning of the prototype.

3. Results and Discussion

The experimentation carried out on figs has shown that for the analysis of large matrices several modifications are necessary to the portable device which currently has the shape of a clamp (therefore capable of completely embracing the small sample).

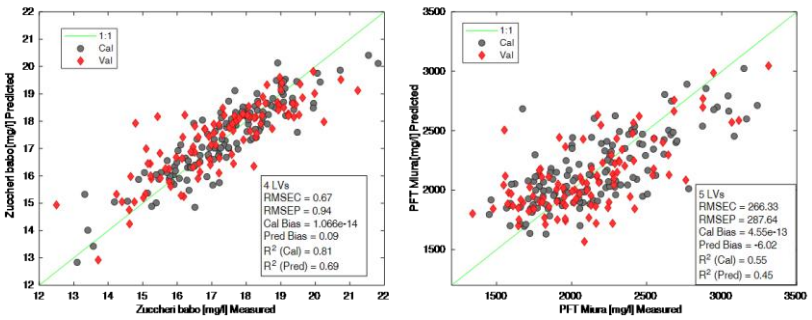

Figure 1. *Results from PLS predictive models on Babo degrees and polyphenol content.*

The experimentation concerning the Ancellotta grape samples has led to good results. Partial Least Square (PLS) regression analysis was applied on grape spectra in order to predict different parameters such as soluble solids content (Babo degrees) and polyphenol content. The results from the PLS models gave coefficients of determination and root mean square errors in calibration and in prediction, respectively, equal to 0.81 and 0.69, and 0.67 (mg/l) and 0.94 (mg/l) for Babo degrees, equal to 0.55 and 0.45, and 266.3 (mg/l) and 287.6 (mg/l) for polyphenols (Figure 1).

The experimentation with the prototype version for liquid analysis initially started to check its performance (using fruit juices of different colours and densities), and then proceeded to identify the parameter setup (integration time and voltage of the halogen lamp) to analyse different types of wine.

From the first tests it was evident how (both with the visible module and with the NIR module) the acquisition is able to give better results in a filtered sample (blueberry sample) which has characteristics more similar in terms of density and colour to those of wine (Figure 2). In the case of must analysis, it will be necessary to carry out filtrations in order to use the prototype.

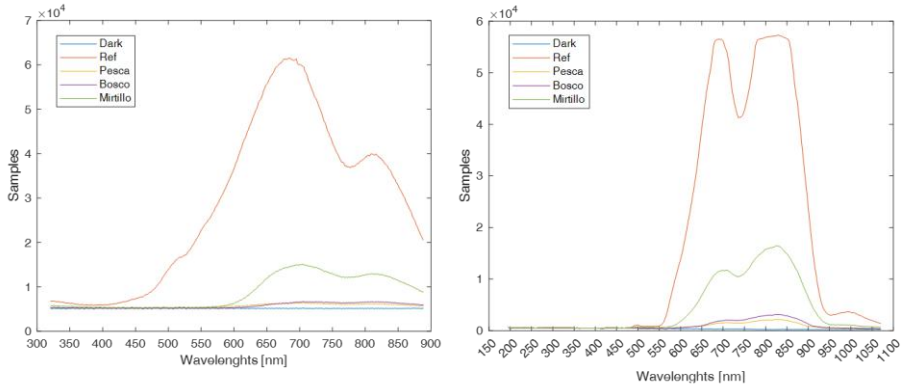

Figure 2. *Optical acquisitions collected with the prototype with the visible module (a) and with the NIR module (b).*

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Development of high-value-oil-based systems through innovative structuring agents and different homogenization techniques for the nutritional and/or functional enhancement of food and non-food matrices

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As first activity of the PhD thesis project, different tree nuts were cold pressed to evaluate the effect of oil extraction on the nutritional properties of derived products and by-products. Different varieties of pistachios, hazelnuts, and apricot kernels were characterized for moisture, ash, protein, fat, tocopherols, and color. They were extracted by hydraulic press giving an oil rich in tocopherols and a defatted cake with high nutrient and functional potential: the cake preserved significant fat, vitamin E, protein, and fiber levels based on the oil extraction yield. Innovative applications of defatted cakes as structuring agent will be investigated.

Sviluppo di sistemi lipidici ad alto valore attraverso l'impiego di agenti strutturanti innovativi e diverse tecniche di omogeneizzazione per il potenziamento nutrizionale e/o funzionale di matrici alimentari e non alimentari

Come prima attività del progetto di dottorato, diversi frutti a guscio sono stati spremuti a freddo per valutare le proprietà nutrizionali dei prodotti e sottoprodotti derivati. Diverse varietà di pistacchi, nocciole e armelline sono state caratterizzate per umidità, ceneri, proteine, grassi, tocoferoli e colore. La pressatura ha originato un olio ricco di tocoferoli e un panello ad alto valore nutrizionale e funzionale. Il panello ha conservato una preziosa quantità di grassi e vitamina E, con un incremento proporzionale di proteine e fibre in base alla resa di estrazione dell'olio. Saranno studiate applicazioni innovative per utilizzare il panello come agente strutturante.

Key words: Tree nuts, oil cold extraction, defatted cake, tocopherols.

1. Introduction

This poster describes the main results of the first activity regarding:

(A1) Select nutritional- and bioactive-rich raw materials;
focus on tree nuts matrices, oils, and defatted cake (DFC) from cold pressing processes, in order to enhance their techno-functional properties and nutritional value.

The next objectives will be:

(A2) Set up greener, milder, and clean label-friendly processes;
formulation of different ingredients and semi-finished products based on fiber-based emulsions suitable for elderly and diabetics.

(A3) Produce high value- oil- based systems (source of proteins, PUFA, tocopherols, and dietary fiber);
evaluation of stability (physicochemical, thermal, rheological, and tribological properties) and food applications.

2. Materials and Methods

Five different three nut kernels (BASE) with the corresponding oils and defatted cake (DFC) were kindly provided by Pariani S.r.l. (Givoletto, Torino, Italy). In detail, samples from two types of hazelnuts (mix of Turkish varieties and Piemonte P.G.I.), two types of pistachios (mix of pistachios varieties from Sicily and Bronte P.O.D.), and a mix of apricot kernels varieties from Turkey were supplied. Pariani's extraction parameters were previously optimized for obtaining the maximum organoleptic evaluation in both DFCs and oils: different kernels were roasted and pressed under different pressure and time allowing to separate oils and DFCs. BASEs and DFCs were preliminary milled and then analysed for colour and moisture, ash, fat, protein, soluble and insoluble dietary fibre, and tocopherols content (Dordoni et al. 2019).

3. Results and Discussion

3.1 Defatted cake characterization

Apricot kernel DFC showed the highest protein concentration 47.63±0.50 due to 79.3% removal of oil. Piedmont P.G.I. hazelnut kernels had the lowest protein content (13.55±0.41% on a d.m. basis) that increased to

38.12±1.42% in DFC, thanks to the highest oil extraction yield 85.6%. In general, the differences in nutritional value between BASE and DFC were primarily due to the redistribution of percentages after the extraction process. Even though the BASEs showed higher protein content on a dry matter basis (26.42±0.21%; 26.93±0.61%), Sicilian and Bronte pistachios had the lowest concentrations among the DFCs. This can be attributed to their lower extraction yields of 50.2% and 38.4%, respectively. Apricot kernels BASE showed a significantly higher moisture content (3.93±0.07%) than other nuts, as they were the only sample that was not roasted but only dried.
The roasting and drying processes generated a decrease in water activity (a_w) bringing all the values to be lower than 0.55. The a_W had a trend that reflected the humidity: the lowest value was Piemonte I.G.P. (0.30±0.01% and 0.40±0.02 for BASE and DFC samples, respectively) and the highest one was for apricot kernel (0.55±0.00 was observed for both the BASE and DFC samples,). From a microbiological point of view, these values represent a safety limit for the growth of bacteria, molds, and yeasts that are unable to duplicate in such conditions (Grant 2004).

3.2 Tocopherol analysis

The tocopherols homologues resulting as the most abundant in the oil and in the lipid fraction of the DFCs were the same which also appeared to be predominant within the BASEs (Table 1). The DFCs of Piedmont and Turkey hazelnuts showed the highest values of α-tocopherol equivalents (α-TE) denoting the best biological activity as antioxidant. DFCs of apricot kernel and Bronte pistachio contained γ-tocopherol values higher than the matrix. The changes in tocopherol concentration and the apparent increase in their total amount (considering DFC and oil contents relative to BASE samples) could be explained as improved extractability due to the pressure effect. Similar results were reported by Ojeda et al. (2018) describing an increase in the concentration of the α-homologue in partially defatted flours, while γ-tocopherol showed a less constant trend.
Alfa-tocopherol resulted to be heat resistant: in particular, Amaral et al. (2006) demonstrated that its concentration remains high even in the Piedmont hazelnut samples subjected to a long roasting treatment at high temperatures. It should be considered that the roasting process is essential to increase aromas, friability, and extraction yield. A screw press allows to extract the oil and simultaneously to heat the sample by developing a toasted flavour, while a hydraulic press requires to roast the product before processing as there is no heat involved in the process. However, by separating the two phases it is possible to ensure greater control over the process and the derived products. DFCs will undergo an evaluation of their functional properties as part of the process of formulating new products.

Table 1: *α-, γ-, β-, δ-tocopherol content (mg/100g of oil) and α-tocopherol equivalents (α-TE) determined on oil, defatted cakes (DFC), and kernels (BASE). Within each column, different letters indicate statistically different values according to post-hoc comparison (Tukey's test) at $p \leq 0.05$. Values are expressed as mean ± sd (n = 6).*

Samples	**Tocopherols (mg/100g of oil)**				
	α	**γ**	**β**	**δ**	**α-TE**
Piemonte P.G.I. hazelnut oil	31.75±2.72e	n.d.	n.d.	n.d.	21.75
Piemonte P.G.I. hazelnut DFC	32.74±1.03e	n.d.	n.d.	n.d.	32.74
Piemonte P.G.I. hazelnut BASE	21.75±2.65cd	n.d.	n.d.	n.d.	31.75
Turkish hazelnut oil	19.95±0.97d	3.03±0.07a	n.d.	n.d.	21.53
Turkish hazelnut DFC	25.34±3.07d	5.19±0.09a	n.d.	n.d.	25.86
Turkish hazelnut BASE	21.00±0.05c	5.30±0.01a	n.d.	n.d.	20.25
Apricot kernel oil	0.27±0.00a	19.80±0.11b	n.d.	1.45±0.03a	3.38
Apricot kernel DFC	0.42±0.02a	30.94±2.17c	n.d.	2.81±0.01b	3.60
Apricot kernel BASE	0.35±0.02a	29.38±2.16cd	n.d.	3.05±0.18c	2.30
Sicilian pistachio oil	1.20±0.02c	22.80±0.02 c	n.d.	1.86±0.04b	6.93
Sicilian pistachio DFC	0.88±0.04b	19.85±0.20b	4.35±0.27a	1.42±0.06a	5.08
Sicilian pistachio BASE	1.00±0.01b	25.51±0.82b	6.66±0.07b	1.54±0.02b	3.54
Bronte P.O.D. pistachio oil	0.86±0.003b	25.66±0.51d	5.39±0.04	1.49±0.03a	6.13
Bronte P.O.D. pistachio DFC	1.52±0.04c	39.53±1.86d	12.46±0.04b	3.97±0.02c	11.82
Bronte P.O.D. pistachio BASE	1.08±0.78b	28.38±0.36c	4.35±1.46a	1.46±0.01a	6.16

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Plant growth promotion and antibiotic resistance of invading bacteria in a plant holobiont perspectives

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The first two-years activities of this PhD focused on the plant holobiont response to the administration of different non-pathogenic invading bacteria considering the plant growth, the shift of the plant microbiome composition and the possible spread of emerging contaminants (i.e., antibiotic resistance genes - ARGs) in agri-food ecosystems. Firstly, the acquisition of ARGs by a plant associated bacterium has been investigated *in planta*. Secondly, the response of micropropagated plants and their endophytic microbiome to the inoculation of putative beneficial strains has been characterized.

Capacità di promozione della crescita vegetale e antibiotico resistenza di batteri invasori dell'olobionte vegetale

Le attività dei primi due anni di PhD si sono focalizzate sulla risposta dell'olobionte vegetale alla somministrazione di differenti batteri invasori non-patogeni, considerando la crescita della pianta, il cambiamento della composizione del microbioma vegetale e la diffusione di contaminati emergenti (i.e., geni dell'antibiotico resistenza) in sistemi agro-alimentari. È stata studiata *in planta* l'acquisizione di geni dell'antibiotico resistenza da parte di un batterio associato alle piante. In un secondo caso studio, sono state caratterizzate le risposte di piante micropropagate e del loro microbioma endofitico all'inoculazione di ceppi batterici descritti come possibili promotori della crescita.

Key words: plant-growth promotion, plant holobiont, antibiotic resistance spread, micropropagation, invasion.

1. Introduction

Plants and their associated microbiota can be defined as 'holobionts' (Trivedi et al., 2020). Till now, bacterial invasion in plants has been largely studied in relation to pathogens, while few data are available regarding this phenomenon in the frame of beneficial (i.e., Plant Growth Promoting strains) or neutral (i.e., antibiotic resistant) plant-bacteria interaction. However, the ability to successfully colonise the plant is one of the major factors limiting the application of microbial biofertilizers and biostimulants in the field, and a better understanding of invasion would benefit sustainable agriculture. Moreover, antibiotic production and/or resistance are key traits for the colonization of environmental niches, including the rhizosphere. Antibiotic resistant bacteria and Antibiotic Resistant Genes (ARGs) can enter agri-food ecosystems by different routes including water reuse after depuration (Christou et al., 2017) and horizontal gene transfer plays a crucial role in the spreading of ARGs (Santala et al., 2016) in several micro-niches, including plant surface. Given the importance of the plant microbiome and beneficial bacteria for food production, and the risk of antibiotic resistance spread through HGT in food systems, I am focusing the research activities of my PhD on the intersections with plant growth promoting and antibiotic resistant bacteria, in compliance with the One Health concept. This will be seen from a holistic approach, considering both the plant and the bacterial community response to the addition of specific bacterial strains.

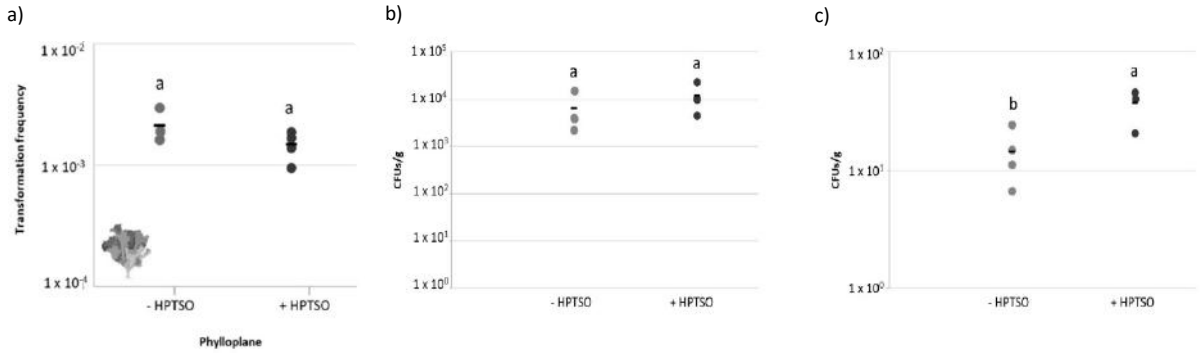
2. Materials and Methods

In the first round of experiments, the environmental strain *Acinetobacter baylyi* BD413 was used to study the acquisition of extracellular DNA (exDNA) carrying an antibiotic resistance gene (ARG) on lettuce phylloplane, performing experiments at conditions (i.e., plasmid quantities) mimicking those that can be found in a water reuse scenario. Moreover, we assessed how the presence of a surfactant, a co-formulant widely used in agriculture, affected exDNA entry in bacteria and plant tissues, besides the penetration and survival of bacteria into the leaf endosphere. Natural transformation of *A. baylyi* BD413 in presence of pZR80 (gfp) plasmid was tested *in planta* using lettuce plants (*Lactuca sativa* var. Canasta) under greenhouse conditions. The experiment was conducted using four replicates, corresponding to four leaves of the same lettuce plant: the four leaves were inoculated with 10^9 cell/mL *A. baylyi* BD413 cell suspension mixed with 10 ng of the pZR80 (gfp) plasmid (final volume of 100 µL). In addition, one leaf was inoculated with cell suspension (no plasmid, negative control) to assess the absence of native kanamycin-resistant bacteria on the lettuce phylloplane. Each bacterized leaf was covered using a sterile empty Petri dish to avoid environmental contamination from the greenhouse. After 24 h, the inoculated leaves were removed from the plant with a sterile scalpel and kept in the Petri dishes, where, after surface-sterilization, we isolated total and transformant *A. baylyi* BD413 colonies from the leaf surface and endosphere. The strain

identity, and the acquisition of the gfp gene was checked by PCR to assess the occurred transformation. Detailed protocols have been published along with the study results by Riva et al. 2022.
In the second case-study, we characterized for PGP activities a collection of endophytic strains established from grapevine and lettuce collected in the field. Among the most promising strains, *Rhizobium* sp. GR12 and *Kosakonia* sp. VR04 were tagged with genes coding for fluorescent proteins and used to inoculate micropropagated grapevine cuttings. Plantlets were grown *in vitro* for three weeks under optimal growth conditions or on a diluted medium to mimic nutritional deficit. Afterwards the plant biomass was measured to evaluate the PGP activity of the strains, and the colonization of the plant tissues was assessed through qPCR amplification of the marker genes from the DNA extracted from plant tissues. We described the endophytic community of micropropagated grapevine plants by integrating high-throughput 16S rRNA gene sequencing and cultivation-based analyses.

3. Results and Discussion

Figure 1 *Influence of heptamethyltrisiloxane on (**a**) the natural transformation of A. baylyi BD413 on lettuce phylloplane, expressed as transformation frequency and (**b**) the strain ability to enter the leaf tissue, shown as total cells and (**c**) for transformed ones. Letters indicate significant differences according to Student's t-test.*



In planta natural transformation experiment was performed to determine the transformation frequency on lettuce phylloplane, in the presence and absence of HPTSO (Figure **1A**). The effect of the tested surfactant on the permeability of *A. baylyi* BD413 cell membrane was assessed in presence of HPTSO. The bacterial cell permeability was not influenced by the presence of the surfactant in the growth medium, coherently with the lack of increased natural competence. Considering that the application of surfactant molecules may enhance the internalization of bacteria into lettuce leaves, by performing the natural transformation experiment in planta we also aimed at measuring the concentration of *A. baylyi* BD413 total and transformant colonies in the lettuce leaf tissues. As hypothesized, the concentrations of total and transformant *A. baylyi* BD413 colonies in the lettuce endosphere showed higher values in leaves treated with HPTSO **(**Figure **1B-C)** and such difference was statistically significant for transformant colonies (p= 0.0149). The latter result could be related to a higher uptake of exDNA by plant tissues in the presence of HPTSO resulting in the occurrence of transformation events directly in the endosphere.
On the other hand, for the second case study, high-throughput 16S rRNA gene sequencing and cultivation-based analyses showed that bacterial endophytic community of grapevine cuttings differed from those generally associated to this plant species in the field. Moreover, the composition of the endophytic community was differently modulated by *Rhizobium* sp. GR12 and *Kosakonia* sp. VR04 and specific taxa were enriched or depleted in response to the invasion by these bacteria, reflecting the different plant response in terms of growth promotion. Our results confirmed the importance of interplays between the plant microbiome members and their dependence upon the plant growth conditions, shedding a light on the previously hidden diversity of endophytic community in micropropagated grapevine plants.

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Investigation on oenological tannins and their role in enhancing the longevity of white wines

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The aim of this PhD research project is finding one or more strategies to help winemakers to produce long-lived white wines. The experimental plan takes into consideration two main stages of the productive process, the pre-fermentative operations, and the fining period. Among all the techniques and additives that can be used in winery, this paper shows the results obtained using oenological tannins. We considered tannins as adjuvants that can be applied as fining agents before and after white wine stabilization. Tannins play an important role in wine quality, they are frequently added during winemaking, but their impact on white wine is poorly documented.

Studio sui tannini enologici e sul loro ruolo nel promuovere la longevità dei vini bianchi

Lo scopo di questo progetto di dottorato è individuare strategie enologiche che possano aiutare l'enologo nella produzione di vini bianchi longevi. Il piano sperimentale prende in considerazione due momenti fondamentali del processo produttivo, le operazioni pre-fermentative e il periodo di affinamento. Tra tutte le possibili tecniche e utilizzi di coadiuvanti, si vuole mostrare i risultati ottenuti dall'analisi dei tannini ad uso enologico. I tannini sono considerati coadiuvanti che possono essere utilizzati sia prima che dopo la stabilizzazione del vino. I tannini al giorno d'oggi rivestono una parte importante nella qualità del vino finale e per questo sono sempre più spesso utilizzati nella vinificazione, ma il loro impatto sui vini bianchi è poco studiato.

Key words: redox evolution, cyclic voltammetry, proteins, polyphenols

1. Introduction

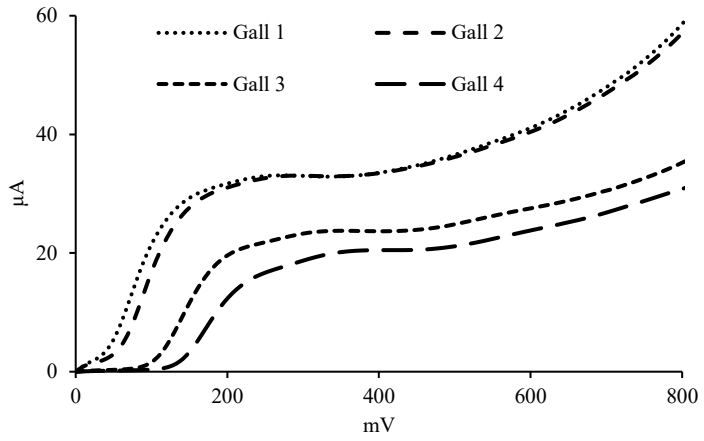
In Europe the use of oenological tannins in winemaking is allowed (Council Regulation (EC) No 1493/1999) and an increasing number of commercial preparations from different origin are available. In this view, the analytical characterisation of oenological tannins is important because they have different chemical and biological activities according to their chemical composition. Oenological tannins are commercial natural products extracted from different botanical sources. This class of natural additives can be broadly classified as either hydrolysable or condensed tannins. The first class includes glucosides, either from gallic acid (gallotannins) or from ellagic acid (ellagitannins), whereas condensed tannins, also called proanthocyanidins, are polymers of flavan-3-ol monomers, such as (+)-catechin, (−)-epicatechin and their gallates (Laghi et al., 2010). The addition of tannin powder to wines during winemaking is a longstanding technological practice in wine industry for different purposes. Considering that oenological tannins are obtained from a wide array of botanical sources with a large diversity of chemical composition, the effects on wine properties are diverse. In fact, some works have shown that oenological tannins should be used with great care; in fact, depending on the characteristics of the tannin extract and its composition and concentration of phenolic substances, their addition sometimes have a negative effect on wine characteristics (Cliff et al., 2012; Harbertson et al., 2012). While the industry relies on tannin for many applications, in white wines the use of oenological tannins aims at improving mouthfeel and body, as well as at protecting wine against oxidation; in fact there are studies for understanding the role of tannins in the redox processes occurring in wine (Magalhães et al., 2014). When we talk about white wine, we must take into consideration the total content of polyphenols (TPC) in fact in white wines the average TPC range between 100 and 300 mg/L (Simonetti et al., 1997). This is important because the use of oenological tannins in white wines can modify severely the structure and the sensory perception of the product. Even a small addition of oenological tannins can have important sensory consequences in poorly structured white wines.

2. Materials and Methods

All the oenological tannins were characterized using the analytical methods described below. Different commercial tannins were supplied by Dal Cin Gildo S.p.A. (Concorrezzo, Italy). The samples were prepared at final concentration of 100 mg/L according to the guideline suggestions. Solutions of each tannin were prepared in a synthetic model wine solution MS (12% v/v ethanol, 5 g/L tartaric acid adjusted to pH 3.2 with sodium hydroxide) and in two types of wine, one supplied by an Italian winery of the north-east (unstable wine, UW) and the other bought from the supermarket (stable wine, SW). All the analyses were carried out at least in triplicate. All the samples were analysed by measuring the UV and visible spectra. Also, the reactivity with wine proteins was measured with the aid of a turbidimeter. At the same time, cyclic voltammetry (CV) profile was acquired using screen-printed electrode (SPCE) model DRP-C110 Metrhom Dropsense (Metrohom Italiana S.r.l., Origgio, Italy) and EmStat pico potentiostat (PalmSens BV, Houten, The Netherlands). Finally, the sensory impact of the different tannins was evaluated in wine solutions.

3. Results and Discussion

3.1 Characterization of different oenological tannins



It is known that UV and visible spectra allow classifying the different tannins based on their botanical origin. however, in winemaking practice, tannins from the same source may give different performances. CV may allow a better characterization of tannins concerning their effect on wine redox potential. In fact, as we can see in Fig. 1, four different lots of gall tannins give four different voltammograms, and this results in a different mechanism of action on white wines.

Fig 1. *Voltammograms of four different lots of gall tannins.*

3.3 Reactivity of oenological tannins with white wine's proteins

The tannin-protein interaction is deeply studied for red wines but when we talk about white wines there are less information. Table 1 shows the different response of the wine after the adding of oenological tannins in stable and unstable wine and the consequent heat treatment. Data can be explained in this way, if the addition is made in an unstable wine, oenological tannins react with the more reactive protein fraction that is already present in wine and the increment of turbidity is consequently higher. Instead, when the oenological tannins are added in a stabilized wine, this phenolic fraction creates a lesser increase of turbidity, and we can say that in this way these adjuvants can exploit their action by regulating redox potential.

	ΔNTU				
	Wine	**Gall**	**Thè**	**Mix 1**	**Mix 2**
UW	20,6	117,8	34,2	74,5	71,8
SW	0,4	-0,2	0,2	7,35	2,2

Table 1. *Differences of NTU pre and post heat treatment of the wines. Wine = UW and SW without tannins*

3.2 Different behaviors of the same oenological tannin in different wines

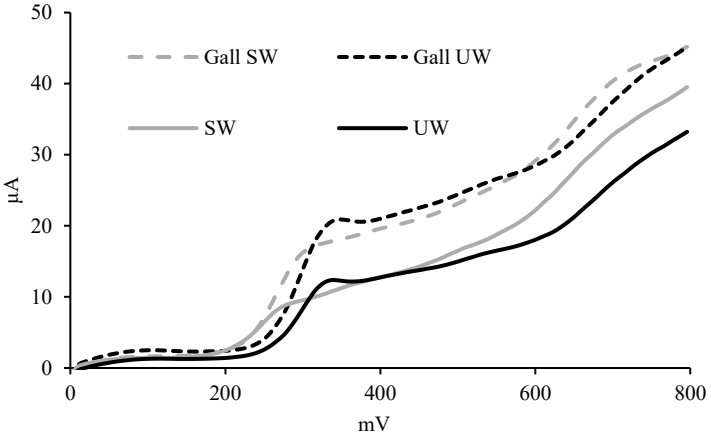


Fig. 2 presents the voltammograms illustrating the behaviour of a particular tannin in various mediums. It is evident from the results that the introduction of gall tannin elevates the potential of the wine, while no noticeable disparity is observed between the two wine types. This observation appears counterintuitive since the presence of abundant unstable proteins in UW would typically lead to a reduction in the curve. However, it is important to note that the absence of a direct correlation at this moment may be attributed to the possibility that a longer duration is required for the tannin to establish binding and effectively fulfil its intended role.

Fig 2. *Voltammograms of the same gall tannins in different mediums.*

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New Strategies to Face Antibiotic Resistance in Healthcare and Food Sectors

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The PhD thesis project aims to develop an electrochemical genosensor to detect specific antibiotic-resistant genes (ARGs) in food matrices. From milk and meat samples *Staphylococcus aureus* and *Escherichia coli* were isolated and tested for resistance against antibiotics. Specific primers for the main ARGs were employed in multiplex PCRs on the isolates. DNA sequences of ARGs were aligned and used for the design of DNA probes, intended for utilization in the electrochemical genosensor.
The isolated strains were tested for their capability of biofilm production to investigate the potential correlation between biofilm formation and antibiotic resistance (AMR).

Nuove strategie per combattere la resistenza agli antibiotici nei settori sanitario e alimentare

Il progetto di dottorato mira a sviluppare un genosensore elettrochimico per rilevare specifici geni responsabili di resistenza agli antibiotici (ARG) in alimenti. *Staphylococcus aureus* ed *Escherichia coli* sono stati isolati da latte e carne e testati per la resistenza agli antibiotici. Primer specifici per i principali ARG sono stati impiegati in multiplex PCR sugli isolati. Le sequenze di DNA degli ARG sono state allineate e utilizzate per la progettazione di sonde per il genosensore elettrochimico.
Gli isolati sono stati testati per la produzione di biofilm per studiare la potenziale correlazione tra biofilm e resistenza agli antibiotici (AMR).

Keywords: Antibiotic resistance (AMR), Antibiotic-resistant genes (ARGs), Electrochemical genosensors, DNA probe design.

1. Introduction

In accordance with the PhD thesis project previously described (Pinamonti, 2022), this poster reports the main results of the microbiological and molecular analyses.
(A1) Microbiological analyses. *S. aureus* and *E. coli* isolation from food matrices and phenotypic characterization. AMR profiling of the isolates.
(A2) Molecular analyses. DNA extraction and PCR confirmation of the species. Selection of specific primers for ARG detection and multiplex PCR on *S. aureus* and *E. coli* isolated resistant and susceptible strains. Design of DNA probes specific for ARGs.
(A3) Biofilm production test. Analysis of biofilm production with classical method.

2. Materials and Methods

A total of 29 samples of raw cow milk and 12 samples of raw milled meat (beef and pork) were analysed.
The bacterial pathogens, *S. aureus* and *E. coli*, were isolated on selective media within 24 hours after milking or purchase. From the countable plate, up to five colonies of each suspected *S. aureus* and *E. coli* were picked up, purified, and subjected to catalase, oxidase, and Gram staining tests.
DNA extraction of the strains followed the classical phenol-chloroform method described by Manzano et al. (2003).
The isolated strains were confirmed using PCR targeting the species-specific thermonuclease *nuc* gene for *S. aureus* and the malic acid dehydrogenase *mdh* gene for *E. coli* (Hsu & Tsen, 2001; Javid et al., 2018).
The confirmed *S. aureus* and *E. coli* strains were tested for antimicrobial susceptibility (AST) following the EUCAST Disk Diffusion test instructions (EUCAST, 2022). Based on the AST results, ARGs were selected and tested for their presence in the isolates. To this aim multiplex PCRs were performed. DNA ARG sequences were used as templates to design DNA probes for the detection of specific ARGs. The probes were evaluated in silico using AmplifX 1.5.4, OligoAnalyzer and BLAST (Basic Local Alignment Search Tool) programs. The specificity of the probes was further confirmed using the dot-blot technique (Cecchini et al., 2012).
Finally, the biofilm production capability of the isolated strains was quantified in microtiter plates, following the method described by Stepanović et al. (2007).

3. Results and Discussion

3.1 Microbiological analyses: *S. aureus* and *E. coli* isolation and Disk Diffusion test for antimicrobial susceptibility test (AST)

In raw milk samples, *E. coli* was detected in concentrations ranging from <1 colony-forming unit (CFU)/mL to $8x10^3$ CFU/mL, while *S. aureus* was found in concentrations ranging from <2 CFU/mL to $7x10^3$ CFU/mL. In the raw meat samples, the concentration of *S. aureus* varied from 2 CFU/g to $4x10^1$ CFU/g in beef and from <2 CFU/g to $1x10^3$ CFU/g in pork meat. On the other hand, *E. coli* was present in concentrations ranging from 1 CFU/g to $5x10^1$ CFU/g in beef and from 6 to $1x10^2$ CFU/g in pork meat.

A total of 71 *S. aureus* strains and 102 *E. coli* strains were isolated from the milk samples, while 33 *S. aureus* strains and 75 *E. coli* strains were isolated from the meat samples. These isolates were confirmed by PCR analysis. The results of the AST for the isolates are reported in Figure 1. The predominant antibiotic resistances among *S. aureus* strains isolated from the food matrices were resistance to tetracycline, erythromycin, and gentamicin. Among *E. coli* strains, the most frequently observed antibiotic resistances were resistance to ampicillin, ciprofloxacin, gentamicin, trimethoprim-sulfamethoxazole, and piperacillin-tazobactam.

3.2 Molecular analyses: multiplex PCRs and DNA probes for antibiotic-resistance genes (ARGs)

The multiplex PCRs detected the presence of tetracycline-resistant genes (*tetK*, *tetL*, *tetM*, *tetO,* and/or *tetS*), as well as macrolide-resistance genes (*ermA*, *ermB*, *ermC*, *msrA*, and/or *mef*), and β-lactam-resistance gene (*mecA*) in *S. aureus* isolates. Additionally, through multiplex PCRs, the presence of β-lactamase genes (*blaTEM*, *blaSHV,* and/or *blaCMY-2*) and sulfamide-resistant genes (*sul1* and/or *sul2*) was confirmed. These findings provide evidence of the presence of specific ARGs in strains that demonstrated resistance to AST.

To confirm the specificity of the designed DNA probes for *tetK*, *tetM*, *mecA*, and *blaTEM* genes, dot-blot tests were conducted. The synthesized and labeled DNA probe with 5'-end digoxigenin demonstrated specificity to *tetK* gene. The specificity of other DNA probes was validated through in silico analysis at this stage.

3.3 Relationship between biofilm production and antibiotic resistance (AMR)

The exploration into the biofilm formation capability of the isolated strains, both *S. aureus* and *E. coli*, has thus far failed to reveal an evident relationship with AMR.

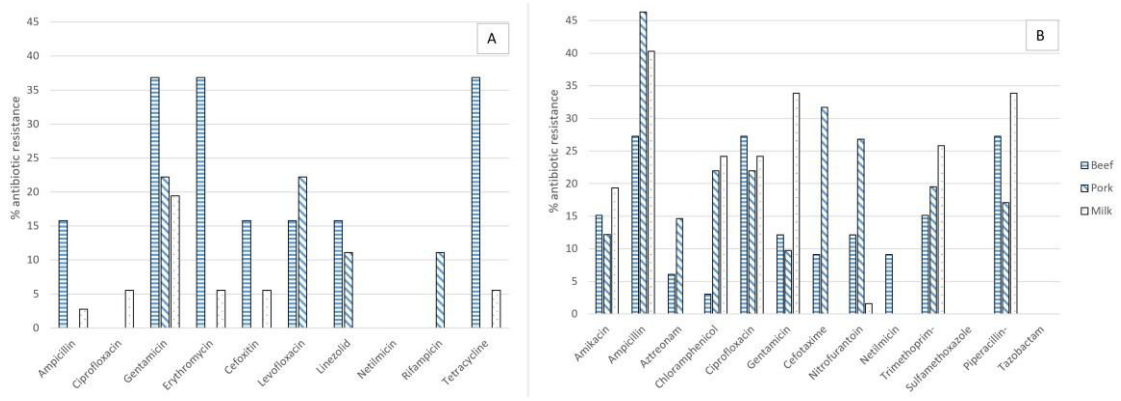

Figure 1 *Percentages of AMR S. aureus (A) and E. coli (B) isolates after Disk Diffusion test*

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Grapevine-associated microorganisms as biocontrol agents against the proliferation of pathogenic fungi

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This PhD project aim at selecting potential biocontrol agents (BCAs) able to prevent, or limit, the infection of grapevine and grape (table and wine) by pathogenic fungi, reducing the use of chemical fungicides. The first performed activities of the project are described. Firstly, the evaluation of endophytic community in *Vitis vinifera* and *V. sylvestris* through set up of protocols for the cultural isolation and biomolecular identification. Secondly, the investigation of BCA-plant interaction through assessment of plant-BCA assays in grapevine cell cultures.

Microorganismi associati alla vite come agenti di biocontrollo contro la proliferazione di funghi patogeni

Il seguente progetto di dottorato mira a selezionare dei potenziali agenti di biocontrollo (BCAs) in grado di prevenire, o limitare, l'infezione di vite e uva (da tavola e vino) da parte di funghi patogeni, in modo da ridurre l'utilizzo dei fungicidi chimici. In seguito, sono descritte le prime attività del progetto svolte. In primo luogo, la valutazione della comunità endofita in piante di *V. vinifera* e *V. sylvestris* tramite messa a punto di protocolli per l'isolamento culturale e identificazione biomolecolare. Successivamente, l'indagine delle interazioni BCA-pianta tramite utilizzo di colture cellulari di vite.

Key words: biocontrol agents, endophytes, grape cell culture.

1. Introduction

In accordance with the PhD thesis project previously described (Pizzi, 2022), this poster reports the main results of the activities concerning:

WP1. Evaluation of endophytic community in *V. vinifera* and *V. sylvestris*:

T1.1 Set up of protocols for the cultural isolation of endophytes (M1.1, Sterilization step optimized)

T1.2 Isolation of culturable microorganisms from grape flowers, leaves and fruits (M1.2, Isolation of at least 10 culturable species)

WP3. Investigation of BCA-plant interaction

T3.1 Set up of protocols for BCA-plant interaction (M3.1 Identification of the best cultivar for grapevine cell cultures)

Nowadays, the following activities are ongoing:

T2.1 Screening of potential biocontrol capability (M2.1 Selection of at least 1 potential BCA against *Botrytis cinerea*)

T2.3 Determination of the mechanisms of action of BCAs (M2.3 Report on the main mode of action of at least 1 potential BCA against *B. cinerea*)

T3.2 Assessment of plant-BCA assays in grapevine cell cultures evaluating the interaction in presence/absence with *B. cinerea* (M3.2 Inoculum and cultural conditions for the preparation of plant-pathogen-BCA validated)

2. Materials and Methods

T1.1 Grapevines located in four locations of the norther Italy were sampled in six vineyards chosen for their different conduction. The sampling was carried out during different phenological stages from April until July 2022. To check the efficacy of surface sterilization for endophytes isolation, a challenge test was performed through contamination with *Saccaromyces cerevisiae EC1118 UMY341* and two protocols of sterilization were evaluated.

T1.2 Endophytic populations were isolated from distinct parts of the plants (i.e., shoots, leaves and berries) and each sample was sterilized with ethanol and sodium hypochlorite, with different pre-treatments depending on the analyzed part of the plant. The shoots were cut in sections and placed with the vascular vessels facing the medium, the leaves were shattered with Bead beater (Biomedicals), and the berries were homogenized with NaCl 0.8%. All plates were incubated at 26°C for one week. Then, all isolates were stored at -80°C in glycerol 20% (v/v). Species identification was performed using the sequencing of taxonomically relevant regions within the ribosomal DNA of bacteria (16S rDNA) and fungi (ITS sequences).

T3.1 For the callus preparation, leaves were sterilized, put on solid Murashige and Skoog (MS) medium supplemented with hormones and maintained at 26°C in darkness for two weeks until callus cells appear. To obtain liquid culture, 2 g of fresh callus was inoculated in the same medium without agar in the same condition of callus. The following activities are on-going:

T2.2 Evaluation of the efficacy of BCAs in controlling the *B. cinerea* infection by culture inhibition tests (dual-culture plate and double Petri dish)

T2.3 Assessment of the biocontrol mechanisms of action of BCAs by VOCs analysis. VOC production will be measured in solid and liquid co-culture with plant/BCA/pathogen contact using SPME coupled with GC/MS analysis.

T3.2 Microscopy observation of the plant/BCA/pathogen interaction in cell cultures and detection of resveratrol production (HPLC) and/or the expression of specific target genes (RT-PCR).

3. Results and Discussion

To evaluate the efficacy of the sterilization step on the samples collected for the endophytes isolation different protocols were applied. Challenge tests, using a contamination with *S. cerevisiae* [$10^7 UFC$/mL], demonstrated that to rinse the sample in 90% ethanol and then in 2.5% sodium hypochlorite with further three washing steps in sterile water, each for 3 min, allowed the sterilization of the material. In accordance with Campisano *et al.* (2014), the same concentration of ethanol and sodium hypochlorite in contact with shoot samples was proved efficient to remove the presence of superficial epiphytes.

A total of 220 endophytes were isolated from grapes, consisting of 72 fungi and 148 bacteria. Specifically, 69 endophytes were isolated from shoots, 120 from leaves and 31 from berries. The results obtained from the species identification are summarised in Table 1.

Table 1 Species identification by sequencing of ribosomal DNA of bacteria (16S rDNA) and fungi (ITS sequences)

Bacteria species identification		**Fungal species identification**	
Bacillus cereus	*Massilia sp.*	*Alternaria alstroemeriae*	*Elsinoe sp.*
Bacillus megaterium	*Microbacterium sp.*	*Alternaria alternata*	*Filobasidium wieringae*
Bacillus velezensis	*Mycobacteroides abscessus*	*Alternaria infectoria*	*Paraconiothyrium brasiliense*
Brevibacillus sp.	*Okibacterium fritillariae*	*Aureobasidium pullulans*	*Plenodomus enteroleucus*
Burkholderia sp.	*Pantoea agglomerans*	*Chaetomium globosum*	*Talaromyces amestolkiae*
Curtobacterium sp.	*Paracoccus yeei*	*Ciboria rufofusca*	
Deinococcus citri	*Pseudomonas coleopterorum*	*Cladosporium sp.*	
Dermacoccus nishinomiyaensis	*Ralstonia pickettii*	*Cryptovalsa ampelina*	
Dermacoccus sp.	*Sphingomonas echinoides*	*Cytospora cedri*	
Enterococcus faecium	*Staphylococcus warneri*	*Diatrype stigma*	
Kocuria sp.	*Stenotrophomonas sp.*	*Didymella pinodella*	
Leifsonia sp.		*Diplodia seriata*	

The bacterial endophyte population usually present in grape is variable because it can depend on different conditions (i.e., environmental, vineyard conduction, grape cultivar). As shown in several studies, the most isolated bacteria belong to the genus *Ralstonia sp., Burkholderia sp., Pseudomonas sp., Agrobacterium sp., Bacillus sp.,* and *Curtobacterium sp.* Similarly, fungal endophytes have been shown to belong to the genus *Alternaria sp., Didymella sp.,* and *Cladosporium sp.* (Campisano *et al.,* 2014, Pancher *et al.,* 2012, Aleynova *et al.,* 2021).

Regarding the ongoing activities, three BCA candidates were chosen to evaluate the activity against two strains of *B. cinerea.* Preliminary results obtained from dual-culture plate and double Petri dish have shown that BCAs are able to reduce the mycelium growth both by direct contact (production of metabolite/proteins/enzyme) and production of VOCs. GC/MS analysis have shown that in a liquid co-culture system, where all components are in direct contact, volatile compounds such as acids, alcohols, and a low quantity of terpenes are released (e.g., benzyl alcohol, butanoic acid, longifolene). Moreover, the microscopy observation has shown that, when placed in co-culture, fungi and BCAs interact directly with plant cells. Finally, a reduction in the expression of genes belonging to the phenylpropanoid pathway in a co-culture system compared with callus/*B. cinerea* control culture has been shown which suggests that BCAs may have inhibitory properties against fungal activity.

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Technological, sensory, and nutritional assessment of eco-friendly food lipids

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The first two activities of the PhD thesis project are described. Firstly, the characterization of different types of refined vegetable oils obtained from different matrices. Said oils were also stored and subsequently analysed following thermal and photooxidative stress. Secondly, the formulation and chemical analysis of different types of baked products, sweet and salty, that were stored for different periods of times, ranging from 0 to 75 days for "tarallini" and 0 to 355 days for "frollini" biscuits.

Valutazione tecnologica, sensoriale e nutrizionale di lipidi alimentari eco-sostenibili

Vengono di seguito descritte le prime due attività del progetto di tesi di dottorato. In primo luogo, la caratterizzazione di diversi tipi di oli vegetali raffinati ottenuti da diverse matrici, di seguito stoccati e successivamente analizzati a seguito di stress termico e foto ossidativo. In secondo luogo, la formulazione e l'analisi chimica di diverse tipologie di prodotti da forno, dolci e salati, che sono stati conservati per diversi periodi di tempo, che vanno da 0 a 75 giorni per i tarallini e da 0 a 355 giorni per i frollini.

Key words: Lipid oxidation, thermal stress, photooxidative stress, product formulation, chemical analysis

1. Introduction

In accordance with the PhD thesis project previously described, this poster reports the main results of the first two activities concerning:

(A1) the characterization of different types of refined vegetable oils obtained from different matrices, highlighting also the behaviour following thermal and photooxidative stress.

(A2) the formulation and chemical analysis of different types of baked products, sweet and salty, that were stored for different periods of times, ranging from 0 to 75 days for "tarallini" and 0 to 355 days for "frollini" biscuits.

2. Materials and Methods

Preceding the methods described, extensive bibliographic research regarding the oil characteristics and production was made. The oil samples analysed in this research work have been acquired, partly through direct purchase in supermarkets premises (CONAD CITY Viale Gaspare Finali, 28, 47521 Cesena FC) and include: Extra Virgin Olive Oil Apruntino Pescarese DOP, Conad 0.75 liters (EVO), 1 liter "Olitalia" grape seed oil (V), 1 liter Conad sunflower oil (BO) and partly through direct supply from private individuals, specifically high oleic sunflower oil (AO). In particular, the analyses carried out on the oil samples were the following: the fatty acid composition b(FAME) by capillary gas chromatography, the peroxide value (PV), the volatile compounds by gas chromatographic analysis combined with mass spectrometry (SPME-GC-MS) (Purcaro et al., 2008), the *p*-anisidine value by spectrophotometric method (p-AV), and the oxidized fatty acids (OFA) by gas chromatographic analysis. The samples (about 10 g) were placed inside of 100 ml bottles with Sovirel caps and placed in an oven at 100°C for different times, as described in table 1. The same oil samples were treated with light (λ 150) for different periods of time, ranging from 0 to 480 minutes. For this experiment, a photooxidation chamber, made in laboratory, were used. The same analyses described for the thermally stressed samples, were conducted for this second test. After the characterization of said oils was conducted, a phase of product formulation came after, using samples normally buyable in convenience stores; said decision was made consequently the delays in the company experience. Five types of "tarallini" and six types of "frollini" biscuits were produced using the recipes highlighted in the tables.

3. Results and Discussion

3.1 Vegetable oil characterization

The oils were analysed and characterized and the results regarding the oxidative parameters are reported in Table

1. In summary both the high and low oleic sunflower oil showed the best attitudes for the baking products conditions, given the high content of relatively stable unsaturated fatty acids (both MUFA and PUFA) correlated to the high contents of α-tocopherol; rice oil showed a good aptitude to temperatures up to 180°C thanks also to the high concentration of γ-oryzanol; grapeseed oil was able to develop unique aromas (following thermal stress) and characteristics thanks to the high concentration of pigments and phenolic substances; finally, extra-virgin olive oil, as expected developed the oxidative parameters more than each other sample, but with values greatly under law limits .

Sample	oil	Treatment time (min)	T° (°C)	Treatment time (min)	Wavelength (λ)
V0	Grapeseed oil	0	100	0	150
V1		30		5	
V2		60		10	
V3		90		20	
V4		120		30	
V5		180		60	
V6		300		240	
V7		/	/	480	
BO0	Low oleic sunflower oil	0	100	0	150
BO1		30		5	
BO2		60		10	
BO3		90		20	
BO4		120		30	
BO5		180		60	
BO6		300		240	
BO7		/	/	480	
AO0	High oleic sunflower oil	0	100	0	150
AO1		30		5	
AO2		60		10	
AO3		90		20	
AO4		120		30	
AO5		180		60	
AO6		300		240	
AO7		/	/	480	
EVO0	Extra virgin olive oil	0	100	0	150
EVO1		30		5	
EVO2		60		10	
EVO3		90		20	
EVO4		120		30	
EVO5		180		60	
EVO6		300		240	
EVO7		/	/	480	

Table 1: *treatment sheet of the vegetable oil samples.*

3.2 Product formulation

Five different lipid formulations for "tarallini" and six different lipid formulations for "frollini" were taken into study. The fat blends used were compromised for each product with one standard formulation using "standard" industrial fats, indicated as 0 (palm oil for "frollini" and extra virgin olive oil for "tarallini"), and the remaining formulations being gradual increasing blends of alternative and industrial fats. All the fats used were not put through specific refining processes given the delays in the expected company period. The products obtained were then characterized, at different shelf-life periods (dark storage at 20°C) by: peroxide value analysis (Shantha N.C. E Decker E.A., 1994), volatile compounds composition by gas chromatography and mass spectrometry (SPME-GC-MS) and accelerated, general oxidation resistance, using an OXITEST® instrument (Riciputi and Caboni, 2017).

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Unlocking the Antioxidant potential of Kamut Wheat: Insights from Triple Detector Analysis of Phenolic Compounds

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This study examined the antioxidant properties of bound and free phenolic extracts from Kamut wheat (*Triticum turgidum* ssp. *turanicum*). Bound extract exhibited superior antioxidant activity, phenolic content, and flavonoid content compared to free extract. Using a triple detector system, the study identified and quantified antioxidant compounds. Phenylalanine, tyrosine, tryptophan, and apigenin 6-C-arabinoside-8-C-glucoside contributed to the antioxidant capacity of the free extracts. The bound extract, after alkaline hydrolysis, contained hydroxycinnamic acids, ferulic acid, and derivatives, demonstrating stronger antioxidant activity. These findings highlight Kamut wheat's potential as a valuable source of dietary antioxidants for the development of functional foods with health benefits.

Svelare il potenziale antiossidante del frumento Kamut: Studio dall'analisi a triplo rilevatore dei composti fenolici

Questo studio ha esaminato le proprietà antiossidanti degli estratti fenolici legati e liberi del grano Kamut (Triticum turgidum ssp. turanicum). L'estratto legato ha mostrato un'attività antiossidante, un contenuto fenolico e di flavonoidi maggiore rispetto all'estratto libero. Utilizzando un sistema a triplo rivelatore, lo studio ha identificato e quantificato i composti antiossidanti, come fenilalanina, tirosina, triptofano e apigenina 6-C-arabinoside-8-C-glucoside. L'estratto legato, dopo idrolisi alcalina, conteneva acidi idrossicinnamici, acido ferulico e derivati, dimostrando una maggiore attività antiossidante. Questi risultati evidenziano il potenziale del grano Kamut come fonte preziosa di antiossidanti alimentari per lo sviluppo di cibi funzionali con benefici per la salute.

1. Introduction

In accordance with the PhD thesis project previously described (Razem 2022), this poster reports the main results of the first two activities concerning: (A1) Extraction of antioxidant compounds from Kamut wheat, (A2) The study of the antioxidant activity and characterization of bound and free antioxidant compounds present in Kamut wheat with a triple detector.

2. Materials and Methods

Kamut wheat samples were obtained from Molino Merano, Italy, and processed by drying, grinding, and sieving. Free phenolic compounds were extracted from defatted flour fractions using methanol (80%), followed by centrifugation and concentration. Bound phenolic compounds were extracted from the remaining free phenolic pellets through alkaline hydrolysis. The total phenolic and flavonoid contents of the extracts were determined using the Folin-Ciocalteu reagent and total Flavonoid assays. The oxygen radical absorbance capacity and DPPH radical scavenging activity were evaluated as measures of antioxidant activity. A triple detector analysis approach was used, which included an HPLC system coupled with a diode array detector (DAD), a CoulArray detector (CAD), and a mass spectrometer (MS) detector. The use of this approach allowed the characterization of bound and free phenolic extracts of Kamut wheat and identified the most potent antioxidant compounds present in each.

3. Results and Discussion

3.1 Antioxidant assays

The primary antioxidants that may contribute to cereals' antioxidant activity are phytochemicals, amino acids, tocopherols, and tocotrienols (Okarter *et al.*, 2010). In the antioxidant assays conducted, the bound phenolic extracts of Kamut wheat showed significantly higher total phenolic content (TPC), total flavonoid content (TFC), and antioxidant capacity compared to the free phenolic extracts. The TPC of the bound extracts was six times higher than that of the free extracts, with values of 1024 ± 55 µmol GAE/100g DW and 169.5 ± 13 µmol GAE/100g DW, respectively. The TPC values of the refined Kamut wheat flour were also higher than those reported in previous studies (Dinelli *et al.*, 2009). Similarly, the bound extracts exhibited more than six times the TFC

compared to the free extracts, with values of 148.8 ± 12 µmol QE/100g DW and 23.8 ± 2.9 µmol QE/100g DW, respectively. Moreover, the bound phenolic extracts demonstrated higher DPPH radical scavenging activity and ORAC values, indicating greater antioxidant capacity. These findings suggest that the bound phenolic extracts of Kamut wheat have superior antioxidant potential, attributed to their higher phenolic content.

Table 1: *Total phenol content, total flavonoid content, DPPH, and ORAC of free and bound phenolic extracts of Kamut wheat.*

Extract	TPC	TFC	DPPH	ORAC
	µmol GAE / 100 g DW	µmol QE / 100 g DW	µmol TE / 100 g DW	µmol TE / 100 g DW
Free Phenols	169.5 ± 13^{B}	23.8 ± 2.9^{B}	46.7 ± 1.8^{B}	767.5 ± 88^{B}
Bound Phenols	1024 ± 55^{A}	148.8 ± 12^{A}	268.4 ± 4.2^{A}	3736 ± 274^{A}

TPC: Total phenolic content; DPPH: DPPH antioxidant assay; TFC: Total flavonoid content; ORAC: Oxygen radical absorbance capacity; GAE: Gallic acid equivalent: TE; Trolox equivalent; QE: Quercetin equivalent; DW: Dry weight. In a column mean ± SD (n=3) that do not share a letter in the superscript are significantly different ($p < 0.05$).

3.2 Characterization of antioxidant compounds using a triple detector system

The HPLC-DAD-CAD-MS^2 analysis was used to characterize the phenolic compounds in the free and bound extracts of Kamut wheat. The DAD displayed all compounds from the extracts of free and bound phenols from Kamut wheat, while the CAD selectively displays peaks with redox activity (Razem *et al*., 2022). The main antioxidant peaks observed in the HPLC-CAD, were subsequently identified based on retention times, and fragmentation patterns using HPLC- MS^2. In the free extracts, four peaks with redox activity were found, tentatively identified as amino acids (tyrosine, phenylalanine, and tryptophan) and a flavonoid (apigenin 6-C-arabinoside 8-C-glucoside). Among these compounds, tyrosine exhibited the strongest antioxidant activity according to the hydrodynamic voltammogram (HDV) analysis. In the bound extracts (Figure 1), 15 antioxidant compounds were identified, with five of them being identified using reference standards, and having ferulic acid and it's derivatives the most distinctive. The antioxidant compounds identified included phenolic aldehydes, phenolic acids, hydroxycinnamic acids, and a flavonoid. The HDV analysis showed that the bound extracts contained more electroactive compounds with lower half-wave potentials, indicating a greater capability for electron transfer compared to the free extracts.

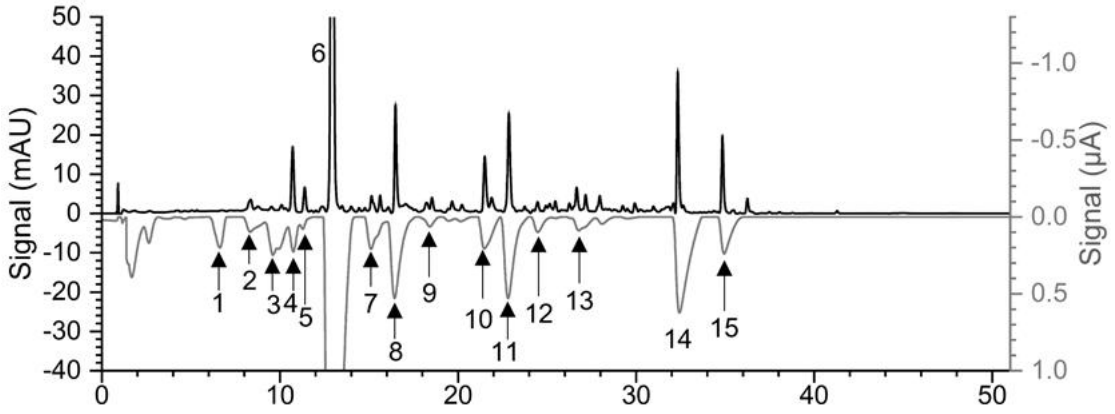

Figure 1: *HPLC-DAD chromatogram of bound extracts of Kamut wheat measured at 280 nm (top chromatogram), and the accumulated sum of current obtained from the 16 CAD channels (bottom mirrorred chromatogram).(1: Protocatechuic aldehyde, 2: vanillic acid, 3: syringic acid, 4: p-coumaric acid, 5: vanillin, 6: ferulic acid, 7: 8-8'-diferulic acid, 8: 8-5'-diferulic acid, 9: quercetin, 10: 8-O-4'-DFA benzo form, 11: 8-O-4'-DFA linear form, 12: 3-4-Dimethoxycinnamic acid, 13: Tri ferulic acid, 14: gamma-tocotrienol, 15: Heliannuol D).*

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Oenological versatility of *Starmerella bacillaris* in white wine

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Fermentation trials were conducted using a *Starmerella bacillaris* CZ31 strain co-inoculated or sequentially inoculated with *S. cerevisiae* RT73 strain, with or without oak chips. Moreover, *St. bacillaris* strain was also tested attached on oak chips. The aim was to better understand the potential of *St. bacillaris* to improve the oenological characteristics of Trebbiano d'Abruzzo wine obtained from *Vitis vinifera* L. cv. Trebbiano Abruzzese grape must.

Potenzialità enologiche di *Starmerella bacillaris* in white wine

Le vinificazioni sono state condotte impiegando un ceppo di *Starmerella bacillaris* CZ31 in co-inoculo o in inoculo sequenziale con un ceppo di *S. cerevisiae* RT73; in presenza o assenza di chips di quercia. Inoltre, il ceppo di *St. bacillaris* è stato adeso alle chips di quercia. Lo scopo è stato di approfondire le potenzialità di *St. bacillaris* in particolare valutare l'influenza sulle caratteristiche enologiche del vino Trebbiano d'Abruzzo ottenuto da mosto d'uva *Vitis vinifera* L. cv. Trebbiano Abruzzese.

Key words: *Starmerella bacillaris*, *Saccharomyces cerevisiae*, wine, adhesion, Trebbiano d'Abruzzo wine

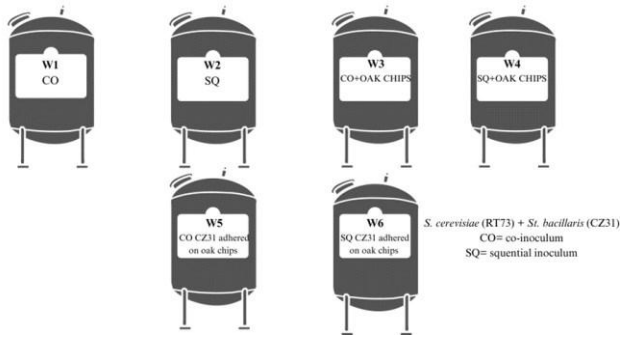
1. Introduction

This poster communication reports the main results concerning the impact of oak chips and *St. bacillaris* in planktonic and sessile states on:

- main oenological physico-chemical parameters
- chromatic traits of the wine
- evaluation of aroma profile

2. Materials and Methods

The strains (*St. bacillaris* CZ31, *S. cerevisiae* RT73) belonging to the Culture Collection of the Microbial Biotechnology Laboratory – Department of Bioscience and Technology for Food Agriculture and Environment (University of Teramo, Italy) were used. Six different vinification trials were carried out in a cellar of Abruzzo region (Italy) and are reported in Figure 1. Briefly, 2 different inoculation strategies were tested: co-inoculation (CO) (trials W1), and sequential inoculation (SQ) (trials W2). Moreover, oak chips (AlcoFermBrew, Poland) (8 g/L) were added in the trials W3 and W4. *Starmerella bacillaris* strain was attached on oak chips as described by Perpetuini et al. (2021) and inoculated in W5 and W6 trials.



Trebbiano Abbruzzese must (sugars 189.6 g/L; pH 3.42; total acidity 5.4 g/L expressed as tartaric acid; volatile acidity 0.12 g/L expressed as acetic acid) was inoculated with a final concentration of 10^6 cells/mL of both yeast strains. The main physical and chemical parameters were monitored through a FOSS WineScan™ FT120. The pH was measured using an InoLab 730 pH meter (WTW, Weilheim, DE). Colorimetric analysis was performed according to the CIEL*a*b* colorimetric standard.

Figure 1 *Fermentation trials performed in this study.*

Organic volatile compounds (VOCs) were detected by solid phase microextraction coupled with gas-chromatography (GC-MS-SPME) as previously described (Perpetuini et al., 2021). All analyses were performed in triplicate. ANOVA test and Principal component analysis (PCA) were performed using XLStat 2014 software (Addinsoft, New York, USA).

3. Results and Discussion

3.1 Determination of the principal phyco-chemical and chromatic characteristics

No significant differences were observed for alcohol, residual sugars, total acidity, and volatile acidity (Table 1). The glycerol content was higher in the wines of the trials W5 and W6. Total polyphenols yield was higher in trials with oak chips (trials W3, W4, W5, and W6). Wines of trials W1 and W2 had a content of polyphenols of 126 mg/L and 183 mg/L respectively, while wines of trials W3, W4, W5, and W6 had a concentration ranging from 220 mg/L (trial W4) to 344 mg/L (trial W6).

Table 1 *Main oenological parameters of obtained wines.*

Trial	Alcohol (% v/v)	Residual sugars (g/L)	pH	Total acidity (g/L)*	Volatile acidity (g/L)**	Glycerol (g/L)	Polyphenols (g/L)
W1	11.07±1.23^{A}	0.51±0.11^{A}	3.48±0.45^{A}	6.05±1.09^{A}	0.56±0.08^{A}	5.19±2.43^{B}	126±37.89^{C}
W2	11.07±2.89^{A}	0.58±0.12^{A}	3.52±0.77^{A}	6.04±1.22^{A}	0.56±0.14^{A}	5.18±1.88^{B}	183±58.43^{C}
W3	11.04±0.98^{A}	0.59±0.09^{A}	3.48±0.26^{A}	6.12±2.09^{A}	0.54±0.06^{A}	5.33±1.05^{B}	223±88.43^{B}
W4	11.13±1.55^{A}	0.61±0.17^{A}	3.56±0.84^{A}	6.11±0.87^{A}	0.52±0.04^{A}	5.15±0.62^{B}	220±91.84^{B}
W5	11.06±2.06^{A}	0.59±0.06^{A}	3.51±0.98^{A}	5.99±0.44^{A}	0.57±0.18^{A}	6.53±1.29^{A}	330±73.54^{A}
W6	11.07±1.22^{A}	0.58±0.04^{A}	3.54±0.37^{A}	6.02±0.35^{A}	0.55±0.09^{A}	6.85±1.84^{A}	344±81.45^{A}

Different letters in the same column indicate significant differences ($p<0.05$)
*expressed as tartaric acid
**expressed as acetic acid

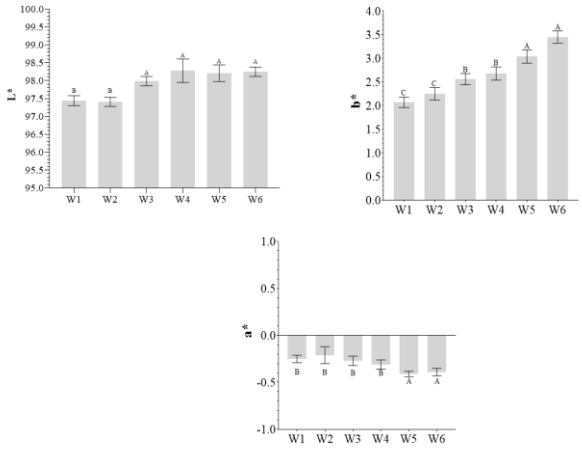


Figure 2 *Effect of S. cerevisiae (RT73) and St. bacillaris (CZ31) strains and oak chips on the chromatic characteristics of Trebbiano d'Abruzzo wines. Different letters indicate significant differences ($p<0.05$)*

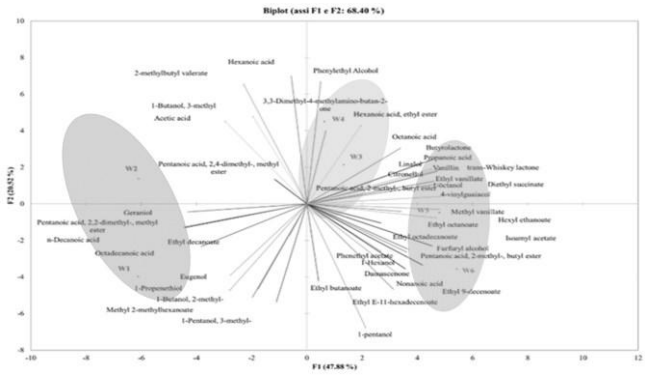


Figure 3 *PCA analysis of the VOCs detected in the different wines*

The presence of chips influenced the color of wines. In fact, the a* values (redness) were all negative values. For b* values (yellowness), the addition of oak chips had shown an increase of the yellow color (Figure 2). Similar results were observed also for c* values and H° angle (data not shown).

3.2 Volatile aroma compounds

A total of 46 compounds have been detected belonging to the following chemical classes: esters (19), higher alcohols (8), organic acids (7), aldehydes (1), terpenes (4), ketones (2), thiols (2), phenols (1), and lactones (2). PCA explained 68.4% of the total variance (47.88% and 20.52% for F1 and F2, respectively) (Figure 3). Wines were differentiated into 3 groups. The first group enclosed the wines fermented without chips (trials W1 and W2); the second group the wines fermented with oak chips (trials W3 and W4) and *S. cerevisiae* and *St. bacillaris* inoculated in the planktonic state, while the third one the oak-treated wines with *St. bacillaris* adhered on oak chips (trials W5 and W6).

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Glycerolysis for Reducing Saturated Fats in Ice Cream

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Although high in saturated fatty acids, tropical fats are commonly used in food industries despite their potential health risks. However, consumers are increasingly seeking sustainable, trans-fat-free, and "clean-label" food options. Accordingly, this Ph.D. project aims to decrease the saturated fatty acid content in fat ingredients through the enzymatic glycerolysis process. The obtained structured lipid was used as a fat source in a mixture for the production of ice cream. Particle size and ζ-potential of the mixture, together with physical properties during melting and textural attributes of the ice cream, were analyzed. Results pave an alternative way to design new fat ingredients with enhanced properties.

Glicerolisi per la Riduzione dei Grassi Saturi nel Gelato

Sebbene ricchi di acidi grassi saturi, i grassi tropicali sono comunemente usati nelle industrie alimentari nonostante i loro potenziali rischi per la salute. Ad oggi, i consumatori sono sempre più alla ricerca di prodotti alimentari, "trans-fat-free" e "clean-label". Questo progetto di dottorato si pone come obiettivo di ridurre il contenuto di acidi grassi saturi in prodotti alimentari, attraverso il processo di glicerolisi enzimatica. Il lipide strutturato ottenuto è stato utilizzato come fonte di grassi in una miscela per la produzione di gelato. Sono state analizzate la dimensione delle particelle e il ζ-potenziale della miscela, nonché le proprietà fisiche e strutturali del gelato. I risultati mostrati aprono un modo alternativo per progettare nuovi ingredienti lipidici con proprietà migliorate.

Keywords: Glycerolysis, structured lipids, low-saturated fat ice cream, high-melting fat.

1. Introduction

Following the Ph.D. thesis project earlier described (Savchina, 2022), the poster reports the main results of the activities:

(A1) Lipid structuring through enzymatic glycerolysis. Where the production of structured fats and optimization of the process were performed.

(A3) Application of structured lipids in a food formulation. Structured fats were introduced into ice cream with a subsequent characterization; explicitly, particle size distribution, ζ-potential, stability while melting, and texture analysis were studied.

2. Materials and Methods

Glycerolysis reactions were performed according to the optimized method described by Nickolson and Marangoni (2020). The main reaction components were peanut oil (10 g), glycerol (1.04 g), and lipase Novozym® 435 immobilized on the beads (0.40 g) were mixed together and placed onto the controlled temperature magnetic stirrer (IKA, USA) at 65°C and 200 rpm speed. After 16 h, the resulting substance was centrifuged (SL 16R Centrifuge, Thermo Scientific, Waltham, MA, USA) at 20 °C at 5,000 rpm for 5 min, and the supernatant was collected as structured lipid (SL). A cream-based ice cream (CB) was prepared by mixing: pasteurized milk cream (81.1 %), sucrose (14 %), and 0.2 % w/w lecithin and guar gum. Two other formulations were prepared by replacing milk cream with an oil-in-water emulsion based on peanut oil (POB) or structured peanut oil (SLB). Whey powder was added to adjust the milk-non-fat content to 5%. The liquid blends were mixed at 75 °C with a laboratory homogenizer (Digital UltraTurrax T25, IKA, USA) at 11,000 rpm for 2 minutes. The resulting blends were mixed with the dry ingredients, cooled down in an ice bath to 4 °C, and aged for 6 h. Aged ice cream premixes were frozen at –10 °C for 20 min at constant mixing in an ice cream maker (Unold 48816, Montereale Valcellina, Italy). Each batch was then sorted in plastic containers and kept at –25 °C for one week for the hardening stage.

The ice cream mixture particle size distribution was determined by a static light scattering technique using a Mastersizer Hydro 3000 (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The sample was dispersed dropwise in deionized water until obscuration values of around 10 % were reached (refractive index of 1.52, absorption index of 0.01). To determine the ζ-potential, the measurements were conducted at a constant temperature of 25 °C after the ice cream blends were diluted (1:1000) with deionized water using a Malvern Zetasizer Nano ZS (ZEN 3600) instrument (Malvern Instruments Ltd, Malvern, Worcestershire, UK).

Turbiscan™ TOWER (Formulation Inc., France) was used to assess ice cream stability while melting. Ice creams were placed in cylindrical glass cells and stored at –18 °C for 24 hours and then placed into the tower at 25 °C for 3 h at 880 nm wavelength scanning. From the backscattering spectra, the Turbiscan stability index (TSI) was calculated and reported as a function of time.

The meltdown test was performed on 50 g ice cream aliquots placed on a plastic grid (3 holes/cm) fitted to drip into beakers. Ice cream blends were kept undisturbed at +23 °C (± 0.5 °C) for 1 h. The first dripping time was recorded, and the melting rate was calculated as a ratio between the melted mass over 60 min time.
A back extrusion test with a 40 mm rig was done to perform the texture analysis of the ice cream using a Texture Analyzer (TA.XT plus C, Stable Micro Systems, UK) fitted with a 50 kg loading cell. Before the analysis, 100 mL plastic cells with frozen samples (–15 °C) were placed onto the measuring plate at 25 °C for 20 min to soften.

3. Results and Discussion

3.1 Ice cream mixtures characterization

Cream-based and structured lipid-based blends did not have significantly different ζ-potential values ($p < 0.05$), opposing to significantly higher ($p < 0.05$) values for peanut oil-based samples demonstrating that the modification in the acylgycerols fractions ratio increased its surface charge. The higher surface charge would, in accordance with prior research, increase the electrostatic repulsion between particles, prevent particle agglomeration, and allow smaller particles to form more stable mixes (Zhu et al., 2019). A similar tendency was observed for the surface mean diameters D[3,2] and the volume mean diameters D[4,3], indicating that the particles of vegetable oil-based ice cream formulation clumped together, increasing their particle size. SLB had a 74 % smaller Dv(90) particle size when compared to POB, which demonstrated that lipid structuring allowed the formation of smaller aggregates due to a minor degree of coalescence among the de-emulsified fat (Roy et al., 2021). The presence of partial acylglycerols decreased the particle size of the SLB sample by 42.53 % compared to the POB, disclosing that the low-polar, non-ionic lipophilic nature of monoglycerides could lucratively inhibit particle aggregation.

3.2 Ice cream textural properties and stability

According to the findings of the texture analysis, lipid structuring ameliorated the firmness, consistency, and cohesiveness of the produced ice cream compared to the peanut oil-based sample. When compared to the CB formulation, the firmness and consistency values of the SLB were not significantly different ($p < 0.05$); however, significantly increased for POB ($p < 0.05$). This denoted that MAG and DAG fractions in SLB samples cross-linked with the water and lipid phase, subsequent in increased matrix stability. The greater firmness values for the POB sample thus could be justified by the development of ice crystals and large aggregates, as also confirmed by the particle size measurements of the mixtures.
Multiple-light scattering technique was used to assess the stability of the ice cream samples over melting at 25 °C. During the 3 h measurement, the TSI values of the POB sample were noticeably higher (up to 48) than those of the other samples, varying between 22–29, implying the progressed stability of SLB ice cream formulations. This could be reasoned by the incidence of MAG crystalline structure formed by the glycerolysis in the SLB sample, which hampered particle aggregation via the creation of a resistant to melting and structural failure system.
Melting is one of the benchmarks when assessing ice cream quality. The melting rate of the PBO ice cream was expressively higher (0.77 ± 0.042 g·min^{-1}) than that of the CB (0.017 ± 0.014 g·min^{-1}) and SLB (0.044 ± 0.021 g·min^{-1}) ice creams. Bearing the same fat content midst the samples, the ice cream made with the SL was more resistant to melting than the sample with the unstructured oil. The bettered melting resistance was attributed to a higher water-binding capacity, provided by an increased emulsification efficiency of the SLB sample compared to the POB ice cream (Góral et al., 2018). Calligaris et al. (2018) have also reported that partial acylglycerides could counteract the lack of solid fat crystals at the surface of air cells, supporting partial coalescence and leading to a structure more able to hold its shape during ice crystals melting. Thus, creating a more stable matrix with a softer texture due to the entrapped air.
The obtained results demonstrated that the studied properties of the ice cream were significantly diminished when liquid oil was used to substitute milkfat in the formulation. This effect is attributed to the absence of solid fat crystals on the air cell surfaces, which limited partial coalescence and made the structure less stable when ice crystals melted (Calligaris et al., 2018). Thus, the experimental results agree with the expected ones, and the present Ph.D. thesis project can be advanced devoid of any significant modification.

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Microbial interactions during fermentations in winemaking

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The early disappearance of non-*Saccharomyces* yeasts in mixed alcoholic fermentations for wine production has not yet been fully explained by classical microbiology. Applying RNA sequencing on yeasts' cells obtained by fermentation in real red grape must should provide insights to the microbial responses occurring in mixed fermentations. Fermentations with *Starmerella bacillaris* and *Saccharomyces cerevisiae* were performed in Nebbiolo must using different conditions (pure, mixed, and mixed with physical separation of cells). Microbiological and chemical analyses data was in line with previous studies. Cells were collected and frozen stored and RNA is going to be extracted with the protocol that has been set up the previous year and sequenced.

Interazioni microbiche durante la fermentazione in vinificazione

La rapida scomparsa dei lieviti non-*Saccharomyces* nella fermentazione alcolica per la produzione del vino non è stata completamente spiegata dalla microbiologia classica. L'applicazione di RNA sequencing sulle cellule di lievito ottenute da fermentazioni in mosto d'uva rossa naturale potrebbe fornire alcune risposte alle modifiche di espressione genica che avvengono durante la fermentazione. Fermentazioni con *Starmerella bacillaris* e *Saccharomyces cerevisiae* sono state svolte in mosto Nebbiolo usando diverse condizioni (in purezza, mista e mista con separazione fisica delle cellule). I risultati delle analisi chimiche e microbiologiche sono in linea con lavori precedenti. Le cellule sono state raccolte, stoccate surgelate e l'RNA verrà estratto con il protocollo messo a punto l'anno scorso per il successivo sequenziamento.

1. Introduction

Many studies demonstrated that the evolution of the must from microbiological and chemical point of view during the fermentation is one of the causes that lead to the early disappearance of non-*Saccharomyces* yeast. It was demonstrated that the concentration of ethanol or other toxic compounds, as the depletion of nutrients are not the unique cause of this behavior (Niessen et al., 2003). Englezos et al. (2019) studied the consequences of the physical contact of *Saccharomyces cerevisiae* and *Starmerella bacillaris* cells in growth dynamics modulation. In case of cells' separation, the non-*Saccharomyces* yeast was able to stay alive longer than in case of contact. But, due to the inability of the classical microbiological tools to investigate the origin of these findings, a transcriptomics approach to study the changes correlated to the physical contact of cells was proposed. RNA sequencing provides insights into the expression of genes, indicating which metabolic pathways are over- or under expressed. To the best of our knowledge, there is a lack of information regarding the study of yeast-yeast interactions involved in mixed fermentation in a real red grape must.

2. Materials and Methods

To simulate the real red wine fermentation conditions, a natural must of Nebbiolo grapes was used. Must was pasteurized at 70°C for 2 hours to ensure the absence of viable microorganisms (confirmed by spreading on WLN medium). Three 500 mL flasks with 200 mL of must and the two compartments of the bioreactor (200 mL each one) separated by a filtering membrane (0.45 µm), were used in this study. All trials were performed in triplicate. The yeasts used were a commercial *S. cerevisiae* (Uvaferm BC, Lallemand, Verona) and two different strains of *Starm. bacillaris* (FC54 and MUT5705); their inoculation concentration was 6.0 Log/mL. The mixed fermentations were performed applying a sequential inoculum of the *S. cerevisiae* after 48 hours. Pure fermentations with each yeast were carried out as control together with two mixed with and without physical contact. Fermentations were conducted at 25°C for 14 days. Samples for RNA extraction were collected at day 2 for pure *S. cerevisiae*, at day 4 for pure *Starm. bacillaris*, at day 2 plus 4 hours for the mixed fermentations, and at day 7 for all; the delay for the pure fermentations has the goal to collect cells from similar must's conditions. Sampling for microbiological and chemical analysis were done at day 0, 2 (for pure fermentations), 2 plus 4 hours (for mixed fermentations), 4, 7, 10, and 14. The RNA extraction will be done using the RNeasy Micro Kit (QIAGEN, Milan). The protocol was optimized during the past year applying some modifications to those proposed by the company.

3. Results

The reported results are those collected until the submission of this manuscript. In pure fermentations, both yeasts overcame 8 Log/mL (at day 2 for *S. cerevisiae* Uvaferm BC and 4 for *Starm. bacillaris* FC54) and then begun to decline, more quickly in case of *S. cerevisiae*. Cells' concentration of *Starm. bacillaris* was higher in both mixed fermentations than in case of pure. In this environment, *S. cerevisiae* was not able to reach the same high concentration as in pure culture. In case of physical contact, the highest concentration was found at day 7 and was lower than 7.5 Log/mL. Plate counts confirmed the quicker disappearance of the non-*Saccharomyces* yeast when involved in mixed fermentation with *S. cerevisiae* compared to the case of physical separation. Differently from previous work (Englezos et al., 2019), *Starm. bacillaris* was not found (<10 CFU/mL) in mixed fermentation in bioreactor at the end point (Table 1). In this case, it was absent at the 14th day while in fermentation conducted in flask it was at the 10th.

Table 1 *Cells count on WLN medium. Values expressed as Log/mL*

	Pure	Pure	Mixed flask		Mixed bioreactor	
Sampling day	*Starm. bacillaris*	*S. cerevisiae*	*Starm. bacillaris*	*S. cerevisiae*	*Starm. bacillaris*	*S. cerevisiae*
D0	6.1 ± 0.2	5.9 ± 0.1	6.1 ± 0.2		6.1 ± 0.2	
D2	7.9 ± 0.1	8.1 ± 0.0				
D2 + 4 h			8.0 ± 0.1	6.2 ± 0.4	8.3 ± 0.2	5.9 ± 0.0
D4	8.1 ± 0.0	7.8 ± 0.1	8.2 ± 0.1	7.2 ± 0.1	8.4 ± 0.2	7.9 ± 0.1
D7	8.0 ± 0.0	7.7 ± 0.0	7.7 ± 0.1	7.4 ± 0.1	8.5 ± 0.4	7.7 ± 0.2
D10	7.9 ± 0.1	7.1 ± 0.3	<1	7.2 ± 0.2	5.3 ± 1.3	7.4 ± 0.5
D14	7.1 ± 0.3	5.8 ± 0.5	<1	7.1 ± 0.2	<1	7.0 ± 0.3

The Nebbiolo grape must used had a concentration of total sugars of 246.39 g/L (glucose 120.37 g/L, fructose 126.02 g/L). *Starm. bacillaris* was not able to ferment all the available sugars (residue of 70.86 g/L mainly represented by glucose) and produced a final amount of ethanol of 11.36 % (v/v). *S. cerevisiae* and the mixed fermentation in flask went dry and produced high amount of ethanol (15.82 % (v/v) and 15.68 % (v/v), respectively), while this didn't happen in mixed fermentation conducted in bioreactor where the highest amount of glycerol (around 10.53 g/L) was produced and ethanol was reduced by about 1 % (v/v), if compared to the control fermented by *S. cerevisiae* in pureness.

4. Discussion

With the goal to study the interactions occurring between a non-*Saccharomyces* yeast and *S. cerevisiae*, transcriptomic analysis is going to be applied on the RNA extracted from the cells collected under different fermentation conditions. The choice of two different days for the first sampling point of cells collection for pure fermentations to have similar conditions of the must was confirmed by HPLC (sugars and ethanol nearly equal; data not shown). The quicker disappearance of *S. cerevisiae* in pure fermentation probably is due to the complete depletion of nutrients. The highest amount of *Starm. bacillaris* cells in mixed fermentation should be correlated to a response due to the competition with another yeast. While *S. cerevisiae* overpassed 8 Log/mL at the second day in pure fermentation, this was not achieved in case of mixed; this should be correlated to factors that reduce its ability to reach high cell concentration. This behavior is more evident in case of mixed fermentation with contact, hypothesizing a disturbance due to the presence of high concentration of a competing yeast (for space and nutrients). As was studied in the past, the physical separation of cells allows a longer permanence of the non-*Saccharomyces* yeast. In this last case, the presence of *Starm. bacillaris* lasted until the tenth day while it was undetectable in case of fermentations with contact. Viable cells of *Starm. bacillaris* was not found at the fourteenth day in mixed fermentation with physical separation. The ethanol concentration (14.97 % (v/v)), that is higher than previous studies done on this couple of yeasts (Englezos et al., 2016), may have played a role in its earlier suppression. The longer presence of *Starm. bacillaris* lowered the ethanol and increased the glycerol (10.53 g/L) as well. It is expected that the transcriptome analysis will lead to a better understanding of the phenomena observed during mixed fermentations.

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Less common grains in bakery industry: product and process optimization

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This paper describes the main research activities conducted in the first 18 months. The aim of the project is to set-up technological and bio-technological processes to enhance the enrichment of baked goods with minor cereals, pseudocereals and/or legumes. Specifically, the main results of the sprouting process applied to oats are here presented. Initially, a literature research was conducted to highlight the knowledge gaps on sprouted oats and to establish the process conditions to be used on a laboratory scale. After that, the effects of sprouting time were studied by assessing the rheological properties of wheat-dough enriched in sprouted oats.

Impiego di cereali minori, pseudocereali e legumi nell'industria dei prodotti da forno: ottimizzazione di prodotto e di processo

Questo lavoro descrive le attività condotte nei primi 18 mesi di dottorato. L'obiettivo del progetto è la messa a punto di processi tecnologici e bio-tecnologici per migliorare i prodotti da forno arricchiti in cereali minori, pseudocereali e/o legumi. Nello specifico vengono qui presentati i principali risultati del processo di germinazione applicato all'avena. È stata dapprima condotta una ricerca bibliografica per evidenziare aspetti ancora poco conosciuti riguardanti l'avena germinata e stabilire le condizioni di processo da utilizzare su scala di laboratorio. Successivamente, sono state valutate le proprietà reologiche di impasti di frumento arricchiti in avena germinata per tempi differenti.

Key words: oats, sprouting time, dough properties.

1. Introduction

This report presents the main results of the 18 months of my PhD project. In accordance with the previously described project for the PhD thesis (Sergiacomo, 2022), this poster reports the main results of the first three activities concerning: (A1) Literature review, (A2) Set up of the process conditions and (A3) Effect of the sprouting on the functionality of blends.

2. Materials and Methods

The literature research was conducted by consulting Web of Science and PubMed electronic databases. The keywords used in the research were as follows: (sprout* OR germinat*) AND (oat) NOT (pre-harvest OR preharvest). After removing the duplicates and excluding the irrelevant records, 26 articles were selected. Commercial dehulled oats kernels were sprouted in a climatic chamber (Memmert, Schwabach, Germany) at 22°C and 90% relative humidity. Seeds were soaked in water (kernels:water ratio of 1:2) for 8 h, sprouted for 48 and 72 h and then dried at 50 °C until moisture content decreased below 14%. Unsprouted oats was used as control. All the samples were grinded to a particle size of less than 250 microns. Pasting properties of unsprouted and sprouted oats were assessed as reported by Suárez-Estrella et al. (2020). Samples were mixed with a commercial wheat flour at 10, 20 and 30% replacement levels. Dough mixing properties were performed by means of the Farinograph-E (Brabender GmbH & Co. KG, Duisburg, Germany) with a 50 g kneading bowl, following the ICC 115/1 Approved Method (ICC, 1992). Three-dimensional extension properties were evaluated with the Alveograph (Chopin, Villeneuve La Garenne, France) following the method AACC 54-30.01 (AACC, 2001).
One-way analysis of variance (ANOVA) followed by Tukey-HSD test ($p < 0.05$) was carried out using Statgraphics Plus 5.1 (StatPoint Inc., Warrenton, USA).

3. Results and Discussion

The literature review (A1) highlighted that most of the studies: (1) investigated the changes in macro and micronutrients during sprouting for 72-96 h; (2) did not investigated the changes occurring at earlier stages of the process and the potential use of oats as ingredient in baked goods. Thus, oats were sprouted for 48 h and 72 h (A2).

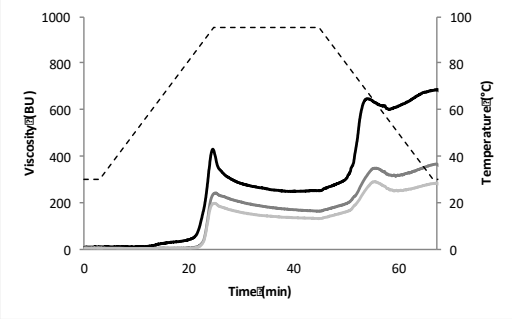

Figure 1 *Pasting profile of unsprouted oats (black line), sprouted oats for 48 h (dark grey line) and 72 h (light grey line). Dotted line refers to temperature profile used during the test. BU: Brabender Units.*

Sprouting decreased the pasting properties of oats, as a function of sprouting time (Figure 1). Specifically, the pasting temperature significantly increased upon sprouting (from 61.8±1.2 °C for unsprouted to 85.5±0.1 and 86.3±0 °C after 48 h and 72 h respectively). Moreover, sprouting decreased the hot viscosity suggesting a limited swelling and gelatinization capacity due to the starch hydrolysis by the amylases developed during sprouting. The decrease in setback values (from 437±7.1 BU for unsprouted oats to 201±1.4 and 149.5±0.1 BU after 48 h and 72 h respectively) upon sprouting suggested a lower ability of starch granules to reorganize during cooling and a lower tendency to retrograde.

As regards the dough rheological properties, by necessity of synthesis only the results related to 20% substitution level will be presented in figures and table.

As expected, replacing wheat with unsprouted oats increased the water absorption (+1-2% as the replacement level increased) and decreased both dough development time (by about 4.5 min) and stability (by about 14 min), likely due to both gluten dilution and protein-fiber interference (Figure 2). To obtain an optimal dough using sprouted oats, a decrease in the amount of water (by 3-4% according to the sprouting time) and mixing time (by 1-4 min based on sprouting time and enrichment level) should be considered.

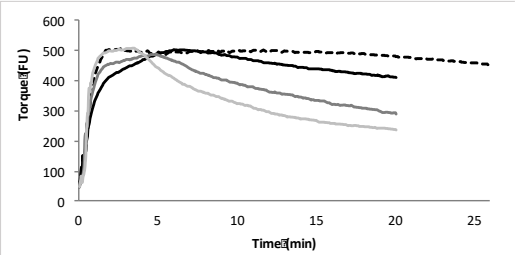

Figure 2 *Mixing properties of wheat alone (black dotted line) and with 20% of unsprouted oats (black line), sprouted oats for 48 h (dark grey line) and sprouted oats for 72 h (light grey line).*

Regarding the dough extensional properties, the addition of oats decreased the dough tenacity (Table 1). The greatest effect was observed when sprouted oats were used; however, neither sprouting time nor enrichment level did affect any further dough tenacity. Considering dough strength, both sprouting time and enrichment level had an effect in dough weakening. Finally, sprouting did not affect the extensibility of oat-enriched dough, whereas the enrichment level did. Overall, sprouting (at 20% substitution level) helped keeping a P/L ratio similar to that of wheat dough.

Table 1 *Extensional properties of wheat alone (WF) and with 20% of unsprouted oats (WF+USO), sprouted oats 48 h (WF+SO48) and sprouted oats 72 h (WF+SO72). Means followed by different letters in the same column are significantly different, according to the Tukey-HSD ($p<0.05$)*

	Tenacity **P** (mmH_2O)	**Extensibility** **L** (mm)	**P/L**	**Strength** **W** ($*10^{-4}J$)
WF	69^{c}±3	115^{b}±14	0.61^{a}±0.1	295^{c}±26
WF+USO	66^{c}±2	76^{a}±4	0.86^{b}±0.03	122^{b}±8
WF+SO48	43^{b}±3	66^{a}±9	0.66^{a}±0.1	63^{a}±9
WF+SO72	31^{a}±1	63^{a}±7	0.49^{a}±0.05	37^{a}±3

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Effect of The Use of Reduced Graphene Modified "Black {001} TiO2" Nanosheets on Ethylene Removal and Quality Attributes of Tomatoes during storage

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The objective of my PhD project is to develop a decision support system (DSS) that can simulate and forecast the shelf-life of fruits and vegetables from distribution to consumption. One of the specific objectives of the work was to study the impact of environmental storage factors on quality indices changes of product. In this context, the aim of this work was to evaluate the performance of a novel synthesized nanocomposite based on reduced graphene oxide (rGO) modified anatase {001} black TiO2 nanosheets (rGO-BTiO2 NSTs) on ethylene removal efficiency and its effect on quality attributes of stored tomatoes.

Effetto dell'uso di nanoparticelle "Black {001} TiO2" modificati con grafene ridotto sulla rimozione dell'etilene e attributi di qualità dei pomodori durante la conservazione

L'obiettivo del progetto di dottorato è quello di sviluppare un sistema di supporto alle decisioni (DSS) in grado di simulare e predire la shelf life di prodotti ortofrutticoli dalla distribuzione al consumo. Uno degli obiettivi specifici del progetto è studiare l'impatto delle condizioni di conservazione sulla qualità del prodotto. In questo contesto, lo scopo di questo lavoro è stato valutare le prestazioni di un nuovo nanocomposito sintetizzato basato su nanoparticele di TiO2 nero anatasio {001} modificato con ossido di grafene ridotto (rGO) (rGO-BTiO2 NST) sull'efficienza di rimozione dell'etilene e il suo effetto sugli attributi di qualità di pomodori conservati.

Keywords: Adsorption, black TiO2 NSTs, ethylene scavenging, photocatalysis, photolysis, postharvest quality, reduced graphene oxide, tomatoes.

1. Introduction

Fresh fruits and vegetables are highly perishable and require proper postharvest handling and storage to maintain their quality and extend their shelf life (Mditshwa et al., 2023). The ripening process of fruits is associated with the production of ethylene, which acts as a signaling molecule to promote ripening and senescence, it stimulates chlorophyll loss, enhances excessive softening, promotes discoloration and browning. Tomatoes are a highly perishable fruit with a relatively short shelf life of 1-2 weeks, depending on factors such as ripeness at the time of purchase, storage temperature, and humidity (Meiramkulova et al., 2023). In this study, we investigated the effect of reduced graphene modified "black {001} TiO2" nanosheets under UV light on ethylene removal and quality attributes of stored tomatoes. The main objectives of the study were: (A1) To evaluate the performance of the new photocatalytic material in removing ethylene and delaying the ripening of tomatoes during storage; (A2) The effect of the photocatalytic treatment on the quality attributes of the tomatoes, such as weight, titratable acidity, soluble solids, moisture content, and lycopene content.

2. Materials and Methods

The tomato plants were cultivated organically in a commercial field plot in Oro Verde (Chillán, Chile). Fruits were harvested at the breaker stage on two different dates (1 and 16 March 2023), and immediately transported to the laboratory and sorted for uniformity according to size and color. 36 fruits were randomly picked from a lot of 70 fruits measured in the values. In addition, 6 randomly chosen fruits were used to find and compare the properties of the raw materials. Tomatoes were stored in hermetically sealed glass desiccators at 12°C in darkness at a relative humidity of 88%. The fruit were stored at 12 °C and 88% relative humidity for 6 days (group 1) and 16 days (group 2) in hermetically sealed 10 L glass desiccators located within a climate incubator (BJPX-A500 II—Biobase Industry, Shandong Co. Ltd., Shandong, China). Four treatments were applied, including a control group, photolysis, adsorption, and photocatalysis. During storage of the tomato fruit, the concentrations of ethylene and carbon dioxide were measured after every two hours. Quality fruit parameters (weight, titratable acidity, soluble solids, moisture content, and lycopene content) were analyzed at the beginning of the experiments, and after 6 days and 16 days of tomato storage.

3. Results and Discussion

3.1 Evolution of Ethylene and carbon dioxide

The effect of different treatments on the evolution of ethylene and carbon dioxide over time can be observed in Figures 1 and 2. The removal of ethylene is minimum in photocatalysis; this can be due that the reduced graphene modified black TiO2 nanosheets having a large surface area, which provides more active sites for ethylene adsorption. The increased surface area also enhances the diffusion of ethylene molecules, which increases the chances of interaction with the active sites on the nanosheets. The photocatalytic activity could involve the generation of reactive oxygen species (ROS) upon light exposure. These ROS species, such as hydroxyl radicals (OH·), are highly reactive and can oxidize ethylene, leading to its decomposition.
Tomatoes undergo photosynthesis in which they utilize light energy to convert carbon dioxide (CO_2) and water into glucose and oxygen. During respiration, they consume oxygen and release CO_2. Black TiO2 is a modified form of titanium dioxide that enhances light absorption capabilities, which makes it an efficient photocatalyst for CO_2 reduction. The use of reduced graphene as a modifier enhances the electron transfer properties of TiO2, leading to a higher efficiency in photocatalytic CO_2 reduction.

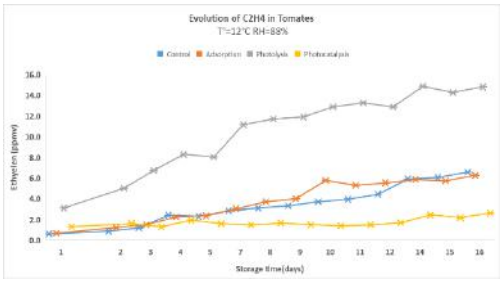

Figure1. *C2H4 evolution during ripening of tomatoes*

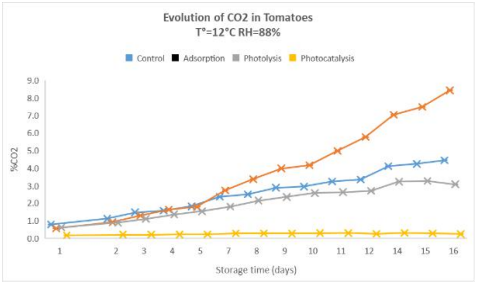

Figure 2. *CO2 evolution during ripening of tomatoes*

3.2 Effect of treatments on tomato quality

Excessive loss of moisture in fresh fruits and vegetables is associated with a negative impact on quality, as a result they can lose their freshness. During our experiment we observed no significant effect of treatments on the weight loss of postharvest tomatoes after 6 days. However, after 16 days, a significant difference was observed (Table 1). The photocatalysis treatment may have slowed down the ripening process and reduced the rate of mass loss compared to the other groups (Li et al; 2022). The application of photocatalysis resulted in a decrease in the total soluble solids (TSS) content of tomatoes after 16 days, while no significant impact was observed after 6 days (Table 1). Maturity Index is a measure used in agriculture to assess the ripeness and quality of fruits and vegetables based on their physical and chemical characteristics (Prasad et al; 2018). In the case of tomatoes, the maturity index is often based on the levels of soluble solids (such as sugars) and acidity (such as citric and malic acid) present in the fruit. This was the most suitable quality parameter for assessing the postharvest performance of tomatoes, due to its increase and significant difference for t = 6 d and t = 16 d (Table 1). The different treatments applied in the experiment had an impact on the lycopene content of the tomatoes, particularly over the 16-day period. The photocatalysis treatment appeared to have a positive effect on the lycopene content of the tomatoes, particularly in the 16-day period. The accumulation of lycopene occurs due to the conversion of chloroplasts into chromoplasts, coupled to the synthesis of this red pigment. The lycopene content of tomatoes correlated positively to both surface and puree colours. This suggests that lycopene content depends upon tomato ripeness.

Table 1. *Physicochemical and ripening indices of tomatoes*

Groups	WL (%)		TSS (%)		MI (%)		Lycopene (mg kg^{-1})	
	6d	16d	6d	16d	6d	16d	6d	16d
Raw Material	-	-	5.6±0.46	4.9±0.63	11.5±1.53	9.4±1.63	4.94±4.94	0.20±0.33
Control	1.5±0.32	5.5±1.41	5.5±0.46	5.1±0.60	12.6±2.60	13.6±3.85	22.69±12.92	9.47±19.65
Catalysis	1.9±0.49	5.8±3.21	5.8±0.52	4.9±0.49	12.8±1.73	11.7±3.38	23.23±13.04	12.82±10.72
Adsorption	1.8±0.66	5.8±3.05	5.8±5.84	4.7±0.83	12.7±2.16	12.0±3.66	22.16±8.97	10.93±14.03
Photocatalysis	1.6±0.34	4.5±1.12	5.6±0.43	4.7±0.60	12.5±1.37	9.8±2.83	24.95±21.60	22.55±11.46

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Improving the grape pressing for a sustainable wine production chain (GrapePress 4.0)

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The first three activities of the PhD thesis project are described. Chardonnay grape samples (vintage 2022) were collected from different vineyards. Firstly, lab-scale must samples were obtained simulating different pressing conditions. Secondly, industrial-scale musts were sampled to investigate the impact of different pressing processes on must composition. Thirdly, winemaking trials were carried out to evaluate the impact of pressing on the characteristics of the resulting wines.

Perfezionamento della fase di pressatura dell'uva per implementare la sostenibilità della filiera enologica (GrapePress 4.0)

Sono descritte le prime tre attività del progetto di tesi di dottorato. Campioni di uva Chardonnay (annata 2022) sono stati raccolti in diversi vigneti. In primo luogo, mosti sperimentali sono stati ottenuti in scala di laboratorio simulando diversi processi di pressatura. In secondo luogo, mosti prodotti in scala industriale sono stati campionati per indagare l'impatto di diversi processi di pressatura sulla composizione del mosto. In terzo luogo, sono state effettuate prove di microvinificazione per valutare l'impatto della pressatura sulle caratteristiche del vino ottenuto.

Key words: Pressing, white grape, base wine, phenols, antioxidant capacity

1. Introduction

In accordance with the PhD thesis project previously (Shanshiashvili, 2022), this poster reports the main results of the first three activities concerning:

A1) Identification of chemical/physical parameters of wine grape.
A1.1 Pressing under lab conditions simulating different pressing processes for the production of musts.
A2) Investigation of the relationship between the pressing cycle on must.
A2.1 Sampling of must on an industrial scale according to pressing conditions adopted by wineries.
A3) Evaluation of the relationship between pressing cycle and wine characteristics.
A3.1 Winemaking trials with minimal intervention approach.

2. Materials and Methods

A1.1. Six Chardonnay grape samples were collected from different vineyards in vintage 2022 in the Franciacorta area (Lombardy, Italy). Lab-scale musts were produced by different pressing conditions: manual press, vacuum press (grape pressing under anoxic condition in vacuum plastic bags), juicer, and small screw press. Moreover, aliquots of whole grape berries were homogenized by an ultra-turrax; for these samples, total and extractable flavonoids were also determined following the procedure described by Di Stefano *et al.* (1989).
A2.1. Must samples were collected at industrial scale following the pressing conditions applied by the winery in terms of both equipment used and pressing cycle applied. The musts were sampled at different extraction yields: free-run juice, 20, 30 [1st fraction], 40, 50 [2nd fraction], 60, and 70 [3rd fraction] % must yields.
A3.1. Experimental base wines were produced from 1st and 2nd fractions following the minimal intervention approach. Musts were inoculated with *Saccharomyces cerevisiae* EC1118 strain (20 g/hL) after the adjustment of readily assimilable nitrogen (RAN) up to 200 mg/L with diammonium phosphate, if required. The fermentations were carried out at 20 ± 2°C in triplicate and daily monitored by weight. At the end of fermentation, wines were racked and stored at 4°C degrees until the in-bottle fermentation. Carbon dioxide (CO_2) release and fermentation efficiency were calculated. The tirage was carried out by inoculating *S. cerevisiae* IOC18-2007 strain (on-going). The parameters determined in both must (lab- and industrial scales) and wine samples were sugars, pH, titrable acidity (TA), tartaric and malic acids, color index (absorbance reading at 420 nm), total phenol index (TPI, Folin-Ciocalteau method), total flavonoids (FLVs). RAN, polyphenol oxidase (PPO), antioxidant capacity (AC), and turbidity units (NTU) were assessed for the must samples. Ethanolic strength, volatile acidity, lactic acid, free and total sulfur dioxide, total dry, and reduced extracts were determined in base wine samples.

3. Results and Discussion

3.1 Lab-scale pressing (A1.1)

Different methods for the production of lab-scale musts were investigated in order to identify the must preparation

mostly representative of the industrial must production. Among the conditions adopted, no difference was found for the major technological parameters usually considered (sugars, pH, TA) (Dumas *et al.,* 2020). On the contrary, the method for must preparation strongly affected the color, AC and phenolic indexes, the latter being compared with the overall quantity determined by means of the homogenized samples. In particular, the highest content of TPI and FLVs were determined in the must samples produced by the juicer, followed by vacuum condition, manual pressing and by small screw press (Table 1). These results suggest the small screw press could be best condition for simulating the industrial pressing.

Table 1 *Chemical parameters of lab-scale must samples*

Samples	**pH**	**Titrable Acidity** (g/L of tartaric acid)	**Color** (AU 420 nm)	**Total phenol index** (mg/L gallic acid)	**Total flavonoids** (mg/L catechin)	**Antioxidant activity** (mM Trolox/L)
Manual press	3.25-3.43	4.68-6.69	0.36-0.72	492-771	46-235	2.26-3.65
Juicer press	3.24-3.57	4.80-6.96	0.98-2.47	915-2169	188-620	5.83-15.0
Vacuum press	3.27-3.55	4.88-6.95	0.40-0.70	366-971	140-343	3.21-3.96
Small screw press	3.18-3.38	4.88-6.91	0.40-0.60	155-342	38-129	1.96-3.69
Homogenized sample	3.34-3.56	N/A	0.54-1.28	629-2195	216-800	7.16-12.0

3.2 Industrial scale pressing (A2.1)

The impact of pressing conditions on must composition was investigated by evaluating the chemical parameters in musts collected at different extraction yields. The content of the phenol-related indexes, NTU, pH, and the color index increased at higher extraction yields, while TA decreased (Table 2). For most of the samples, RAN were lower for increased extraction yields and PPO seemed to be unaffected by the must extraction yield (data not shown). These results can indicate a relevant relationship exists between the different pressing conditions and must characteristics. The phenol-related indexes should be considered in addition to chemical parameters to make the proper decision of the pressing condition being adopted related to the specific characteristics of grape, as well.

Table 2 *Chemical parameters of industrial-scale must samples*

Samples	**pH**	**Titrable acidity** (g/L of tartaric acid)	**Color** (AU 420 nm)	**Total phenol index** (mg/L gallic acid)	**Total flavonoids** (mg/L catechin)	**Antioxidant activity** (mM Trolox/L)
Free-run juice	2.64-3.46	4.9-6.9	0.138-0.389	150-333	32-106	1.8-5.35
20 % yield	3.21-3.51	5.2-7.0	0.214-0.490	143-394	19-119	1.74-5.48
1st fraction	3.02-3.51	5.1-8.6	0.212-0.891	181-314	26-117	1.46-2.91
40 % yield	3.28-3.45	3.37-6.7	0.367-0.760	164-267	35-126	1.88-2.09
2nd fraction	3.29-3.52	5.2-6.0	0.279-0.811	207-483	50-236	1.40-7.80
60 % yield	3.31-3.70	3.8-6.8	0.250-0.856	124-361	26-134	2.07-3.33
3rd fraction	3.53-3.63	4.5-5.8	0.956-1.580	293-470	52-277	3.11-6.20

3.3 Winemaking trials (A3.1)

Alcoholic fermentation (AF) was completed in all the must samples within 8-9 days. CO_2 release and fermentation efficiency differed between the two must fractions fermented. Lower amount of CO_2 was released from AF of the 1st fraction musts and a slight difference in fermentation efficiency was observed (Table 3). The base wines from 1st fractions had higher TA and tartaric acid content, while the base wine from 2nd fraction showed a higher pH and color index (Table 3). Negligible difference was found in TPI content among the base wines from the two must fractions (Table 3).

Table 3 *Fermentation parameters (CO_2 release and efficiency) and chemical parameters of experimental base wines*

Samples	**CO_2 release** (moles/4 L)	**Fermentation efficiency**	**pH**	**Titrable acidity** (g/L of tartaric acid)	**Tartaric acid** (g/L)	**Color** (AU 420 nm)	**Total phenol index** (mg/L gallic acid)
1st fraction	53.3-97.4	57-146	3.00-3.47	5.9-8.4	2.13-4.59	0.11-0.16	7.45-14.0
2nd fraction	43.6-131.3	42-140	3.19-3.72	4.4-6.3	1.92-2.64	0.11-0.81	5.60-14.5

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Valorization of industrial bread waste using enzymatic treatment and sourdough fermentation

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Puratos Italia S.r.l. Supervisor: Marco Ampollini

This PhD project aims to develop a protocol for recycling of bread waste through a combination of enzymatic hydrolysis and sustainable, low-cost and green biotechnology of fermentation, recently rediscovered as a feasible alternative to enhance the technological, nutritional, sensory and functional features of agro-food by-products. "Bread slurries", obtained by mixing bread waste flour and water, were enzymatically hydrolyzed by proteolytic and amylolytic enzymes, alone and in combination followed by fermentation with *Lactiplantibacillus plantarum* and *Saccharomyces cerevisiae* as starters.

Valorizzazione degli scarti di pane mediante trattamento enzimatico e fermentazione con lievito madre

Questo progetto di ricerca è finalizzato al recupero degli scarti di pane attraverso la combinazione dell'idrolisi enzimatica e della biotecnologia low-cost, green e sostenibile della fermentazione, recentemente riscoperta come alternativa per recuperare e/o migliorare le caratteristiche tecnologiche, nutrizionali, sensoriali e funzionali dei sottoprodotti agroalimentari. Gli "impasti di pane", ottenuti mescolando farina di scarto di pane e acqua, sono stati idrolizzati con enzimi proteolitici e amilolitici, da soli e in combinazione, seguiti da fermentazione con *Lactiplantibacillus plantarum* e *Saccharomyces cerevisiae* come starter.

Key words: bread waste, enzymatic hydrolysis, sourdough fermentation, sustainability

1. Introduction

Nowadays, one of the most important categories of food waste is represented by leavened baked goods, such as bread. Over the last decade, many researchers have attempted to find recycling alternatives (Verni et al., 2020). According to the PhD thesis project previously described, this poster reports the main results obtained from the enzymatic hydrolysis and fermentation of the "bread slurries". The optimal amount of water and bread waste flour, type of the enzymes (alone or in combination) and their concentrations, type of starters and time - temperature needed for the enzymatic treatment and fermentation, were determined as the main process parameters.

2. Materials and Methods

White wheat bread crusts were ground and mixed with an optimal distilled water ratio, as previously described (Verni et al., 2021), and homogenized with a blender to obtain "bread slurries", that were subjected to enzymatic hydrolysis and fermentation for 24 h at 30°C. Four enzymes, alone and in combination, were used to hydrolyze bread slurries: glucoamylase (AMYL), amylase fongique (FONG), protease (PROT) and amylase-protease mix (MIX). *Lactiplantibacillus plantarum* and *Saccharomyces cerevisiae* were used as starters for the fermentation. The enzymatically hydrolysed and fermented bread slurries were evaluated for microbial growth (Log CFU/mL) and acidification kinetics, total titratable acidity (TTA), as well as sugars (maltose, glucose, and fructose), organic acids (lactic and acetic acids), proteins, peptides and free amino acids (FAA) concentrations. Consequently, the best performing sourdough in terms of microbial cell density, acidification and lactic acid production was subjected to bread making trials. Different percentages of the prepared sourdough, i.e., 10%, 20%, 30% and 50% were used for breadmaking. All the breads were characterized for pH, TTA, leavening capacity, texture, specific volume, alveolation, and color.

3. Results and Discussion

To select the "enzyme + microorganism" combination that shows the fastest acidification at the end of 24 h fermentation, the growth kinetics of lactic acid bacteria and yeasts in the individual bread slurries, with or without enzymatic hydrolysis, was determined using the Gompertz equation, as modified by (Zwietering et al., 1990)

$$y = k + Aexp\{-exp[(\mu \max e/A)(\lambda - t) + 1]\} \quad (1)$$

where A is the cell density variation (between inoculation and stationary phase); μmax is the maximum growth rate expressed as units/h and λ is the length of the lag phase measured in hours. As reported and highlighted by the circle in Figure 1, bread slurries fermented with *L. plantarum* and *S. cerevisiae* and enzymatically hydrolyzed with protease and amylase-protease mix showed the highest cell densities for both lactic acid bacteria and yeasts than the corresponding fermented slurries without the enzymatic treatment.

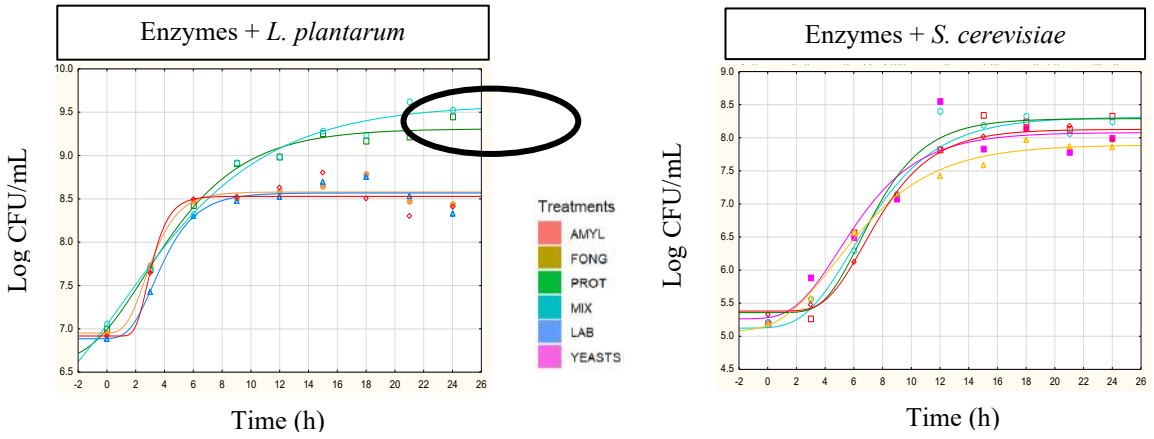

Figure 1. *Growth kinetics of lactic acid bacteria and yeasts alone and in combination with the four different enzymes*

The highest cell growth (Log CFU/mL) of lactic acid bacteria and yeasts in these two samples was also confirmed by the one-way ANOVA with Post-hoc Tukey's comparison ($P < 0.05$). Accordingly, a faster acidification in the "protease + *L. plantarum*" and in the "mix amylase-protease + *L. plantarum*" samples was observed, in accordance with a more than twofold increase in lactic acid concentration compared to the other treated bread slurries.
Therefore, the protease enzyme was selected for the preparation of two different sourdoughs, with the aim of evaluating the effect of combining lactic acid bacteria and yeast: protease + *L. plantarum* (7 Log CFU/mL) and protease + *L. plantarum* (7 Log CFU/mL) + *S. cerevisiae* (5 Log CFU/mL). The quantification of sugars in the sourdough by HPLC analysis revealed that the presence of *S. cerevisiae* caused the depletion of glucose, fructose, and maltose after 24 h fermentation, in agreement with the literature (Paramithiotis et al., 2006), whereas in the "protease + *L. plantarum*" sourdough (where *S. cerevisiae* is not present), maltose was not metabolized.
Finally, the two sourdoughs from bread waste were used in different percentages for bread making trials. The evaluation of the leavening capacity and acidification of the final bread doughs demonstrated the differences among bread doughs prepared using different sourdoughs as well as among bread doughs prepared using the same sourdough but different inoculation percentages of the latter.
It was observed that the specific volume of the final bread decreased with the increasing percentage of sourdough. More specifically, when 10% and 50% sourdough (prepared with protease + *L. plantarum)* was used, the specific volume of the final bread was 2.53 ± 0.02 mL/g and 1.64 ± 0.04 mL/g, respectively, whereas when 10% and 50% of sourdough (prepared with protease + *L. plantarum* + *S. cerevisiae)* was used, the specific volume of the final bread was 2.23 ± 0.16 mL/g and 1.87 ± 0.02 mL/g, respectively.
Therefore, in combination with texture and sensory analyses of these breads, our results indicated the application of enzymatically treated sourdough as a baking ingredient, thus enabling the recycling of bread waste in a sustainable and low-cost circular economy concept.

4. Future perspectives

All the chemical-physical-sensorial parameters of the bread will be considered to define the best fermentation conditions and inoculum percentage. For the next activities, the enzyme concentration for the enzymatic treatment, the protease activity of the bread waste sourdough and the type of starter cultures for sourdough fermentation will be taken into account with a view to further optimise the process.

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Antioxidant Efficiency and Oxidizability of Mayonnaise by Oximetry and Isothermal Calorimetry

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The effect of vegetable oils on the resulting rate of autoxidation in mayonnaise samples was studied by oximetry and isothermal calorimetry at 60°C. The two methods were highly correlated (R^2 = 0.99), showing similar onset times (i.e., antioxidant capacity, τ), and rates of inhibited (R_{inh}) and uninhibited (R_{uni}) periods. The mayonnaise samples showing the highest resistance against peroxidation (i.e., highest τ and lowest R_{inh}) were those prepared with extra virgin olive, followed by corn > grapeseed > sunflower > apple seed oil, whereas the *A.E.* was maximum for sunflower oil. Most importantly, isothermal calorimetry allowed the simultaneous measurement of up to 24 samples, with minimal experimental effort.

Efficienza antiossidante e ossidabilità della maionese mediante ossimetria e calorimetria isotermica

L'effetto degli oli vegetali sulla risultante velocità di autossidazione nei campioni di maionese è stato studiato mediante ossimetria e calorimetria isotermica a 60°C. I due metodi erano altamente correlati (R^2 = 0,99), mostrando tempi di insorgenza simili (cioè capacità antiossidante, τ) e tassi di periodi inibiti (R_{inh}) e non inibiti (R_{uni}). I campioni di maionese che hanno mostrato la più alta resistenza alla perossidazione (cioè, τ più alto e R_{inh} più basso) sono stati quelli preparati con olio extravergine di oliva, seguiti da mais > vinaccioli > girasole > olio di semi di mela, mentre l'A.E. è stato massimo per l'olio di girasole. Ancora più importante, la calorimetria isotermica ha consentito la misurazione simultanea di un massimo di 24 campioni, con uno sforzo sperimentale minimo.

Keywords: Induction time; Fatty acids; Antioxidant activity; Lipid oxidation; Oxidation rate.

1. Introduction

In accordance with the PhD thesis project, this poster reports the main results of the first activity concerning: A1 - the oxidation kinetics of mayonnaise by oximetry and isothermal calorimetric analysis.

2. Materials and Methods

Mayonnaise samples were prepared by mixing oil (80%), egg yolk (10%) and vinegar (10%). Sodium azide (0.05% w/w) was added as microbial inhibitor. To control the rate of free radical formation a lipid soluble radical initiator AIBN was added in the oil phase to reach a final concentration of 25 mM. Samples were labelled according to the oil type used, as MSO, MCO, MEVOO, MGO and MAO, respectively, for sunflower, corn, extra virgin olive, grapeseed, and apple seed oils. To determine the oxidative stability, mayonnaise samples (200±5 mg) were kept in hermetically sealed glass ampoules (4.0 cm3) and heat flow over time was recorded using an isothermal calorimeter (Thermal Activity Monitor, Model 421 TAM III, TA Instruments) at 60°C. In addition, the concentration of oxygen inside the glass ampoules was monitored with an oxygen meter (Fibox 4, PreSens GmbH, Germany) at 60°C. A typical workflow was used to transform the isothermal calorimetric heat flow to oxygen concentration, which was then used to determine the onset time (τ, antioxidant capacity), rate of inhibited period (R_{inh}), and rate of uninhibited period (R_{uni}). The oxidizability of mayonnaise was determined based on R_{uni}. Additionally, antioxidant efficiency (*A.E.*) was calculated using τ and R_{inh}.

3. Results and Discussion

Figure 1 (A) shows the calorimetric traces obtained for the analysis of five different mayonnaise samples, each prepared with the same water phase, but with different plant-based oils. Figure 1 (B) shows the transformation of the calorimetric trace into the corresponding oxygen consumptions. From Figure 1 (B), it was possible to determine the induction time (τ) for the oxidation of each mayonnaise samples, as well as the rates of oxygen consumption during the inhibited (R_{inh}) and uninhibited (R_{uni}) periods.

3.1 Oxidizability

Oxidizability index *(O.I.)* of mayonnaise samples was determined based on eq. (1).

$$O.I. = \frac{k_p}{\sqrt{2k_t}} = \frac{R_{uni}}{[\mathrm{RH}]_0 \cdot \sqrt{R_i}} \qquad (1)$$

Where, R_{uni} is the rate of oxidation during uninhibited period, $[RH]_0$ represent the molar concentration of lipid substrate and R_i is the rate of initiation.

The mayonnaise sample with the highest *O.I.* – i.e., the ones with the highest susceptibility toward oxidation were apple seed oil and sunflower oil mayonnaise with no statistical significant difference ($p < 0.05$), followed by grape seed oil > corn oil > extra virgin olive oil (Table 1). The *O.I.* values were correlated with the content of unsaturated fatty acids ($R^2 = 0.99$). This correlation can be expected considering that the rate constant for a termination reaction, $2k_t$, is similar among different oxidizable substrates (~10^7 $M^{-1}s^{-1}$) (Baschieri *et al.*, 2019), whereas the values for the propagation rate constant k_p is greatly dependent on the degree of unsaturation in the fatty acids (Xu *et al.,* 2009). Moreover, the k_p values that can be calculated from *O.I.* and a $2k_t$ value of 10^7 $M^{-1}s^{-1}$ is of the same order of magnitude (Table 1) as that determined in homogeneous system by oximetry technique, as k_p of pure methyl linoleate is 62 $M^{-1}s^{-1}$.

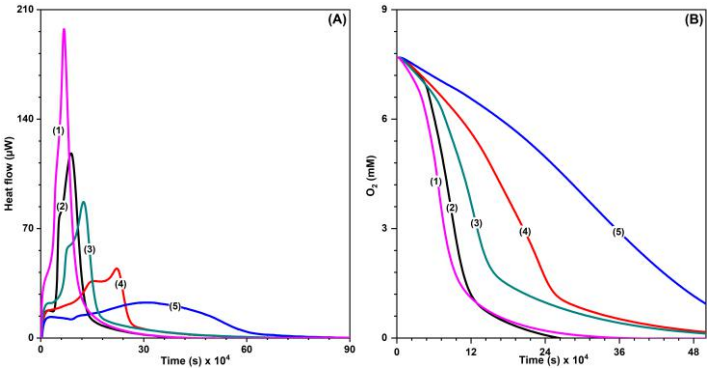

Figure 1 *(A) Isothermal calorimetry trace of mayonnaise at 60°C, (B) oxygen consumption derived from heat flow data. (1) MAO, (2) MSO, (3) MGO, (4) MCO and (5) MEVOO.*

3.2 Antioxidant Efficiency

The concept of "antioxidant efficiency" is far more helpful and practical for quantitatively describing the effects of antioxidants in inhibiting lipid peroxidation (Bravo-Díaz, 2022). The *A.E.* of mayonnaise samples was expressed with eq. (2) (Pryor *et al.*, 1993). As R_i was constant for all the mayonnaise samples, *A.E.* can be simply expressed using the onset time (τ) and R_{inh}.

$$\text{A. E.} = \frac{k_{inh}}{k_p} = \frac{R_i}{n \cdot [\text{AH}]_0} \cdot \frac{[\text{RH}]_0}{R_{inh}} = \frac{[\text{RH}]_0}{\tau \cdot R_{inh}} \qquad (2)$$

Based on the results reported in Table 1, mayonnaise prepared using sunflower oil showed the highest *A.E.* value, followed by apple seed oil > grapeseed oil > extra virgin olive oil > corn oil with no significant difference ($p < 0.05$) between extra virgin olive oil and corn oil. The *A.E.* of sunflower oil antioxidants in mayonnaise was found to be approximately 2.5 times that of extra virgin olive oil and corn oil, and 1.8 and 1.5 times that of grapeseed and apple seed oils, respectively.

Table 1 *Kinetic parameters derived using the isothermal calorimetry trace of mayonnaise samples at 60°C.*

Sample	R_{inh}	R_{uni}	*O.I.*	k_p	τ	*A.E.*
	10^{-7} mol/L.s	10^{-7} mol/L.s	10^{-3} (mol/L)$^{-1/2}$s$^{-1/2}$	(mol/L)$^{-1}$ s^{-1}	10^4 s	-
MSO	2.3±0.1^c	14.2±1.1^a	8.7±0.6^a	29	5.4±0.1^d	198.1±6.6^a
MCO	2.8±0.1^b	5.2±0.2^c	3.2±0.1^c	11	11.8±0.9^b	74.8±4.4^d
MEVOO	1.6±0.1^d	2.5±0.1^d	1.7±0.1^d	5.7	18.8±0.4^a	77.4±2.9^d
MGO	2.9±0.1^b	8.8±1.5^b	5.4±0.9^b	18.3	7.8±0.1^c	108±4.6^c
MAO	4.1±0.3^a	14.8±0.2^a	8.9±0.1^a	30	4.8±0.2^d	128.6±10.5^b

In a column means±SD that do not share a superscript letter are significantly different ($p < 0.05$).

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Development of field-effect transistor-based platforms for the detection of foodborne hazards

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My PhD project aims to develop sensors for detecting foodborne hazards, specifically focusing on remnants of fertilizers, such as ammonium ions, and bacterial byproducts like ammonia and biogenic amines. The first part of the project involved developing an electrolyte-gated carbon nanotube field-effect transistor (EG-CNTFET)-based biosensor functionalized with a selective membrane using the actinomycete ionophore nonactin for ammonium detection. To enhance stability and reduce stabilization time, a lipophilic membrane was added to the biosensors.

Sviluppo di piattaforme basate su transistor ad effetto di campo per il rilevamento dei rischi alimentari

Il progetto di dottorato mira a sviluppare sensori per rilevare rischi alimentari, concentrandosi in particolare su tracce di fertilizzanti, come gli ioni ammonio, e sui sottoprodotti batterici come l'ammoniaca e le ammine biogene. La prima parte del progetto ha previsto la creazione di un biosensore basato su electrolyte-gated carbon nanotube field effect transistor (EG-CNTFET) con una membrana selettiva, utilizzando lo ionoforo di actinomiceti, nonactin. Per migliorare la stabilità e ridurre il tempo di stabilizzazione, ai sensori è stata aggiunta una membrana lipofilica.

Key words: Ammonium, Biogenic Amines, Ion-selective membrane, Lipophilic membrane, Biosensor

1. Introduction

Electrolyte-gated carbon-nanotube field-effect transistors (EG-CNTFETs) are successful biosensing platforms that are able to amplify signals, improving sensitivity and detection limits (Makowski, Ivanisevic 2011). They operate at low voltages, allowing the detection of biomolecules in food without water electrolysis. Early detection of food intoxicants is crucial to prevent outbreaks. Ammonium, found in fertilizers used on crops, requires tight regulation due to potential adverse effects when ingested (Li et al. 2018). Additionally, bacterial amino acid metabolism in protein-rich foods can produce ammonia and biogenic amines, like histamine, causing a range of unpleasant symptoms when consumed in high quantities (Franceschelli et al. 2021, Santos 1996). This poster presents the development of a stable EG-CNTFET biosensor for detecting ammonium in food. Future objectives include histamine sensing, mathematical model evaluation, and computational simulations for validation.

2. Materials and Methods

A planar EG-FET device (Figure 1a) was fabricated by means of standard single-step negative photolithography followed by evaporation of 10nm titanium and 50nm gold; single-walled CNTs were then deposited on the channel using a spray-coater. The protocol was previously described (Shkodra et al. 2022, Shkodra et al. 2021). Atomic Force Microscopy was then conducted to verify the uniform and dense distribution of the CNTs. Later, a lipophilic membrane was prepared and deposited through drop-casting (Joshi et al. 2018). A second membrane was prepared, to functionalize the gate electrode with the nonactin ionophore, to make it selective for ammonium (NH_4^+) detection (Guinovart et al. 2013). The transfer characteristics (source-drain current, I_{DS}, vs gate-source voltage, V_{GS}) were registered in 40 cycles to test the stability of the device: the curves were registered by sweeping the V_{GS} from 0.2V to -0.8V, keeping the drain voltage fixed at -0.1V. The stability was evaluated with 200µl 1x PBS, we tested the performance by adding increments of 20µl of NH_4^+ every 10 minutes to test the ability of the device to detect the analyte at 0.01mM, 0.1mM, 1mM, 10mM and 100mM in 0.1x PBS.

3. Results and Discussion

3.1 Stability of the devices

First, we had to optimize the volume and thickness of the deposited membrane: the desired thickness of membrane was found to be reached by dropcasting 8+7µl of membrane in two separate steps, leading to an average thickness of 80µm. The devices encapsulated by the lipophilic membrane were found to stabilize on average in 34 minutes (see Figure 1b, inset), which is close to half the time of the state-of-the-art devices (Molazemhosseini et al. 2021). Moreover, these devices were able to improve their performance over time, compared to the bare devices (with no encapsulation), whose performance worsened over time, eventually reaching a 0µA current.

3.2 Ammonium detection

The gate electrode of the device was functionalized with an ion-selective membrane based on the nonactin ionophore to detect NH_4^+. The resulting biosensors were found to be able to detect the NH_4^+ analyte in lab conditions at all concentrations tested, from 0.01mM to 100mM. The biosensor's calibration curve in Figure 1c shows that the device is able to detect the analyte with a coefficient of determination of 94.71%, with a response of 0.143 µA/decade.

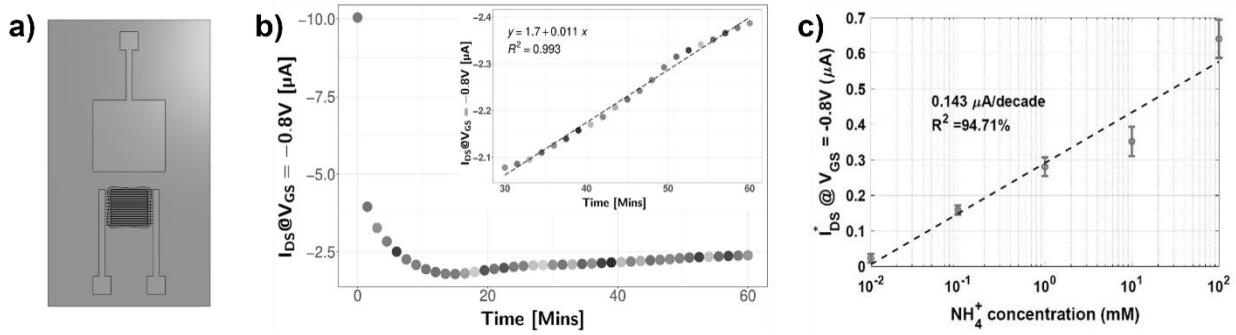

Figure 1 *a) Structure of the EG-CNTFET device; b) I_{DS} at -0.8V collected for each round of transfer: the current initially drops and subsequently increases in linear form. The linearity of I_{DS} over time is reached after 30 minutes (inset of b), i.e., this specific device shows an increase of 11 nA/min with a coefficient of determination of 99.3%; c) Calibration curve of the biosensor for NH_4^+ detection. The sensor exhibits good linearity over the range of concentrations tested, with sensitivity of 0.143 µA/decade and coefficient of determination 94.71%.*

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Study of microencapsulation technologies for the development of powdered food-grade hop extracts as innovative ingredient

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The first activities of the PhD thesis project are described. Firstly, hop cones were subjected to solid-liquid ultrasounds assisted extraction by the use of a food-grade hydroalcoholic solvent. Secondly, hop extracts were microencapsulated by freeze-drying and spray-drying using two different coating materials (Maltodextrin and Arabic gum). The microencapsulated hop extracts were characterised for their chemical properties (moisture content) physicochemical properties (water activity, solubility, flowability), and encapsulation efficiency was evaluated through the retention of α-acids, β-acids and antioxidant compounds.

Studio di tecnologie di incapsulamento per lo sviluppo di estratti di luppolo in polvere da utilizzare come ingrediente innovativo

Vengono descritte le prime attività del progetto di tesi di dottorato. I coni di luppolo sono stati inizialmente sottoposti ad una estrazione solido-liquido assistita da ultrasuoni con l'utilizzo di un solvente idroalcolico ad uso alimentare. Successivamente, gli estratti sono stati microincapsulati mediante liofilizzazione e essiccazione per atomizzazione utilizzando due diversi materiali strutturanti (maltodestrina e gomma arabica). Le polveri microincapsulate sono state caratterizzate per le loro proprietà chimiche (umidità), fisico-chimiche (attività dell'acqua, solubilità, resistenza allo scorrimento) e l'efficienza di incapsulamento valutata attraverso la ritenzione del contenuto di α-acidi, β-acidi e composti antiossidanti.

Keywords: hop extracts; bioactive compounds; microencapsulation; freeze-drying; spray drying, gum arabic; maltodextrin

1. Introduction

In accordance with the PhD thesis project previously described (Fddff, 2022), this poster reports the main results of the following activities:
(A1.1) production of food-grade hop extract by ultrasounds assisted extraction
(A2.1) extract's characterization and antioxidant activity evaluation
(A3) production and evaluation of microencapsulated hop powders obtained by freeze drying and spray drying

2. Materials and Methods

Microencapsulation was carried out by using two different hop-concentrated extracts, obtained from hop cones (cv. Herkules) of two different years, 2021 and 2022 (CE_{2021} CE_{2022}) (P.A.B. S.r.l. Mr. Malt, Pasian di Prato-Udine, Italy) according to the extraction method reported in Santarelli et al. (2022). Briefly, 0.2 g of ground cones were added to 10 ml of hydroalcoholic solution (ethanol: water 50:50 v/v) and treated by ultrasounds (100 Watt and 50 kHz) for 30 min using an ultrasound bath (Falc Instruments, Treviglio, Bergamo, Italy). After centrifugation, hop extracts were concentrated by a rotary evaporator (Buchi R-100) at 45 °C and characterized for: total phenolic content (TPC) expressed as gallic acid equivalent (GAE) per g of concentrated extract; antioxidant activity (AOA) evaluated by ABTS assay (µmoles of Trolox equivalents,TEAC) per g of concentrated extract; and bitter acids content (total α-acids and total β-acids) expressed as %w/w (Santarelli et al.,2022).
Two microencapsulation technologies based on solvent removal, freeze-drying (FD) and spray-drying (SD) were carried out on both hop extracts. Before formulation, CE_{2021} was resuspended in a 0.02% w/w Tween 20 solution, while CE_{2022} in a 5% (v/v) ethanol solution. Thus, the resuspended hop extracts were formulated with Maltodextrin (MD) and Arabic Gum (GA) at 12% w/v using an extract: wall material ratio of 1:9. The FD was carried out using a Labogene (Allerød, Den-mark) Scanvac Coolsafe freeze-dryer set up at 0.316 hPa, increasing the temperature of the shelves from −40 °C to 17 °C in 24 h. SD was performed using the Büchi mini Spray Dryer B-290 (nozzle diameter of 0.7 mm, inlet temperature: 150°C; feed rate: 7.5mL/min; aspirator: 100%). The resulting microencapsulated hop powders (FD_MD, FD_GA, SD_MD and SD_GA) were then characterized by water activity, moisture content, solubility, and flowability using the Carr Index (CI), TPC, total α-acids, total β-acids and ABTS assay according to Tatasciore et al. (2023). Results were expressed as load yield (Y%) of polyphenol (TPC Y%), total α-acids (α Y%), total β-acids (β Y%) and antioxidant activity (AOA Y%). For each analysis, Y% was calculated as the percentage ratio between the initial values determined in the concentrated hop extracts and

the theoretical values determined in the microencapsulated extracts. Data were reported as the mean and standard deviation of three independent measurements and additionally analyzed by one-way ANOVA using STATISTICA for Windows (StatSoftTM, Tulsa, OK, USA) software. Significant differences were calculated by the Tukey (HSD) test at a significance level of $p < 0.05$.

3. Results and Discussion

3.1 Chemical characterization of concentrate hop cones

CE_{2021} and CE_{2022} showed respectively a TPC of 158 and 111 GAE g^{-1} dm and a TEAC of 757 and 635 µmol g^{-1} dm. As for bitter acids, a content of 40% w/w of α-acids and a 7.5% w/w of β-acids was determined in CE_{2021} while 27% w/w of α-acids and 6% w/w of β-acids were found in CE_{2022}. Total phenolic content and the antioxidant activity of hop extracts were similar or higher than other spices and plant foods considered to be rich sources of antioxidant compounds (Pellegrini et al. 2006).

3.2 Physicochemical characterization of encapsulated hop extracts

In Figure 1 moisture content and water activity of the differently microencapsulated hop extracts were reported. All the samples were characterized by very low water content and water activity values. The lowest contents were found in the powders obtained by SD. Among the FD powders, the sample containing Arabic gum showed the highest ($p<0.05$) moisture content and a_w values. Powders' solubility was higher than 99% irrespective of the carrier and the microencapsulation technology. In terms of CI, according to the classifications reported by Jinapong et al.(2008), FD powders are characterised by bad flowability with CI values between 39 and 46 while SD powders showed fair flowability with values between 21 and 23.

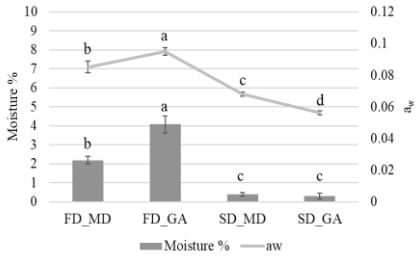

Figure 1 *Moisture content, and water activity (a_w) of differently microencapsulated hop extracts. FD_MD: freeze-dried hop extract with maltodextrin; FD_GA: freeze-dried hop extract with Arabic gum; SD_MD: spray-dried hop extract with maltodextrin; SD_GA: spray-dried hop extract with Arabic gum. Data with different letters are statistically different at p level < 0.05.*

In Figure 2, α Y% and β Y%(a), TPC Y% (b), and AOA Y% (c) are reported. Observing the results (Fig. 2a), in general, values ranged from 50% to 80% and from 30% to 60% for α and β-acids, respectively. Irrespective of microencapsulation technology highest values of α-acids and β -acids were retained when Arabic gum was used as wall material. Polyphenols load yield (Fig. 2b) ranged from 60 to 90. Among all samples, SD_MD showed the highest TPC Y% while no significant differences were highlighted in the other samples. The same trend was observed for AOAY% (Fig.2c). The combined use of SD technology with MD as carrier material enabled to produce hop powders with the highest TPC Y% and AOA Y% while the highest retention of bitter acids was achieved when GA was used as wall material with both FD and SD microencapsulation technology. This result could be due to the higher solubilization and dispersion degree of the bitter acids, promoted by the emulsifying properties of Arabic gum (Yadav et al. 2006).

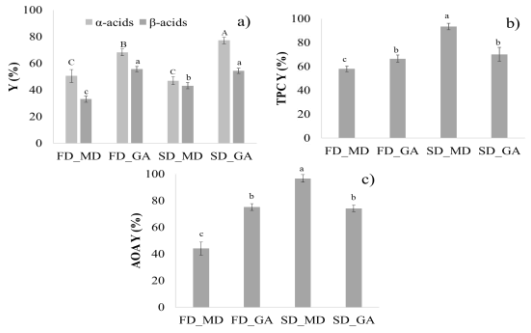

Figure 2. ***a)*** *α and β acids load yield;* ***b)*** *TPC load yield;* ***c)*** *AOA load yield of differently microencapsulated hop extract. FD_MD: freeze-dried hop extract with maltodextrin; FD_GA: freeze-dried hop extract with Arabic gum; SD_MD: spray-dried hop extract with maltodextrin; SD_GA: spray-dried hop extract with Arabic gum. Data with different letters are statistically different at p level < 0.05*

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Use of chitosan from sustainable sources to reduce the sulphur dioxide use in wine production

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The first three activities of the PhD thesis project are described. Firstly, a screening for SO_2 and chitosan resistance was performed among 65 non-*Saccharomyces* strains, belonging to species present in vinification, such as *Metschnikowia pulcherrima*, *Zygosaccharomyces bailii*, *Torulaspora delbruekii*, *Hanseniaspora* spp., *Lachancea thermotolerans*, *Candida zemplinina*, *Pichia* spp. In the second step, selected strains were tested for chitosan resistance in laboratory scale fermentation, whereas in the third step the same strains were evaluated for resistance to commercial and insect-based chitosan on agarized grape must medium.

Utilizzo del chitosano da fonti sostenibili per ridurre l'impiego dell'anidride solforosa nella produzione del vino

In questo lavoro vengono descritte le prime tre attività del progetto di tesi di dottorato. In primo luogo, è stato effettuato uno screening di resistenza alla SO_2 e al chitosano tra 65 lieviti non-*Saccharomyces*, appartenenti alle specie presenti in vinificazione, come *Metschnikowia pulcherrima*, *Zygosaccharomyces bailii*, *Torulaspora delbruekii*, *Hanseniaspora* spp, *Lachancea thermotolerans*, *Candida zemplinina*, *Meyerozyma caribbica*, *Pichia* spp. Nella seconda fase, i ceppi selezionati sono stati testati per la resistenza al chitosano in fermentazione su scala di laboratorio, mentre nella terza fase gli stessi ceppi sono stati valutati per la resistenza al chitosano commerciale e da insetto su terreno a base di mosto d'uva agarizzato.

Key words: Chitosan, resistance screening, non-*Saccharomyces* yeasts, antimicrobial activity, sustainable sources.

1. Introduction

In accordance with the PhD thesis project previously described (Tedesco, 2022), this poster reports the main results of the first three activities concerning:

(A1) the resistance screening to 150 mg/L of SO_2 and 100 mg/L of commercial chitosan among non-*Saccharomyces* yeast strains, belonging to UNIBAS yeast collection (University of Basilicata – Italy) and including the species most frequent in winemaking;

(A2) the laboratory scale fermentations in pasteurised grape must, inoculated with selected strains, and added with 50 mg/L of SO_2; 100 mg/L of chitosan and 20 mg/L of SO_2 + 100 mg/L of chitosan;

(A3) the resistance tests on agarized grape must, added with 100, 200, 300 and 400 mg/L of both commercial and insect-based chitosan.

2. Materials and Methods

In the first screening, sixty-five non-*Saccharomyces* yeast strains were tested. The resistance test was carried out in 96-well microtiter plates, containing the Yeast Nitrogen Base medium (Capece et al., 2020), added with 150 ppm of SO_2 and 100 ppm of chitosan. The inoculum level for each strain was set at $1x10^6$ cells/mL and the strain growth level was followed by OD_{600} evaluation.

On the basis of the first screening, twenty-two yeast strains were selected and tested in laboratory scale fermentations (inoculum level of $1x10^4$ cells/mL) using pasteurized grape must, added with the antimicrobial compounds. The tested conditions were the following: (a) 50 mg/L of SO_2, the amount frequently used during cellar fermentations; (b) 100 mg/L of chitosan, the amount authorized by the OIV (oiv-eno-338a-2009); (c) 20 mg/L of SO_2 and 100 mg/L of chitosan, in order to try to reduce the use of sulphur dioxide; (d) without antimicrobial compounds (positive control); (e) without inoculum and antimicrobials (negative control). In order to study the influence of antimicrobials on the inoculated non-*Saccharomyces* strains, the kinetic of fermentation and the yeast viability were monitored after 48 h of incubation.

The same selected yeasts strains were tested for plate resistance, using agarized grape must added with the following doses of commercial and insect-based chitosan 100; 200; 300 and 400 mg/L. The growth level was monitored after 48 h of incubation.

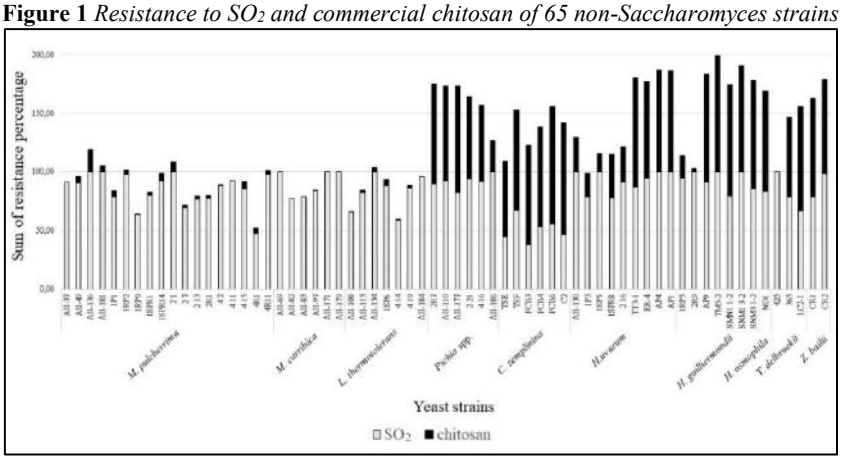
3. Results and Discussion

3.1 Preliminary screening

The resistance level to SO_2 and chitosan was determined by the ratio between strain growth in broth with and without the antimicrobial compounds. The test showed that the assayed non-*Saccharomyces* strains exhibited a

high variability to sulphur dioxide and chitosan tolerance. Strains like *M. pulcherrima*, *Mey. caribbica*, *L. thermotolerans* showed high susceptibility to chitosan, while the others are more resistant to both antimicrobial compounds, although a strain variability was observed (Fig. 1).
Based on the obtained results, twenty-two strains were selected, by choosing those chitosan-sensitive and SO_2-resistant and SO_2-sensitive and chitosan-resistant.

Figure 1 *Resistance to SO_2 and commercial chitosan of 65 non-Saccharomyces strains*



3.2 Chitosan resistance of selected strains

The resistance level of selected strains during lab-scale fermentations were calculated monitoring the cells growth level after 48h of incubation. Resistance to tested antimicrobials was calculated as the ratio between the number of generations in grape must with and without the antimicrobials. The results showed that, in some cases, the chitosan has a higher antimicrobial activity than SO_2 (i.e. *Metschnikowia*) and vice versa. In few cases, the better action is given by the combined use of both, as reported in Table 1.
As the evaluation of chitosan resistance on agarized medium, the tolerance level was reported as the maximum doses allowing the strain growth. The results showed that strains exhibit different levels of chitosan resistance, and, in some cases, the use of a lower dose of insect chitosan showed better antimicrobial activity (Tab. 1).

Table 1 *Strain resistance to antimicrobials evaluated in fermentation (reported as percentage of survival to antimicrobials) and as growth on agarized grape must (expressed as the highest tolerated dose)*

Strain	Species	Grape must fermentation			Agarized grape must	
		SO_2 (%)	Chitosan (%)	SO_2 + chitosan (%)	Commercial chitosan (mg/L)	Insect chitosan (mg/L)
AII-136	*M. pulcherrima*	17,9	18,7	16,2	200	200
4-11	*M. pulcherrima*	74,9	19,4	44,8	200	200
4R1	*M. pulcherrima*	75,4	37,1	29,3	200	200
CR-1	*Z. bailii*	92,4	83,2	80,8	300	200
CR-2	*Z. bailii*	100	100	89,5	400	300
425	*T. delbruekii*	72,5	48,2	48,5	400	400
LC2-1	*T. delbruekii*	93,8	74,5	96,5	400	400
AP1	*H. uvarum*	0	81,1	16,7	200	300
1P3	*H. uvarum*	76,9	100	100	300	300
2R9	*H. guilliermondii*	23,3	100	86,2	200	200
TM5-2	*H. guilliermondii*	6,4	59,1	60,4	200	300
ND1	*H. osmophila*	56,3	82,2	73,3	400	400
AII-134	*L. thermotolerans*	22,3	100	59,4	300	300
4-14	*L. thermotolerans*	41,9	80,2	58,4	300	300
TSE	*C. zemplinina*	90,5	100	100	300	300
FCB6	*C. zemplinina*	52,2	100	78,9	400	400
AII-171	*M. caribbica*	59,1	29,4	29,9	100	100
AII-82	*M. caribbica*	91,5	59,2	33,1	200	100
AII-177	*P. kudriavzevii*	74,5	87,4	75,5	400	400
4-16	*P. kudriavsevii*	61,6	85	79,1	400	400
AII-110	*P. kluyveri*	66,5	68,6	83,8	400	400
AII-186	*P. anomala*	76	83,2	84	200	200

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Yeast dynamics and volatilome analysis of naturally fermented Taggiasca black and green table olives

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In the framework of this PhD project, the activities carried out included: the isolation and molecular identification of yeast colonies from 21-22 harvest to determine the ecology of fermented olives by using culture-based methodologies; 26S amplicon sequencing through the Illumina MiSeq platform to overcome culture-dependent limitations, and to deeply describe fermented olives mycobiota. Microbial ecology data was also complemented with the determination of volatile compounds via Gas Chromatography/Mass Spectrometry (GC/MS) analysis of both brines and olives samples.

Dinamica dei lieviti e analisi del volatiloma di olive Taggiasche da tavola verdi e nere fermentate naturalmente

Nell'ambito di questo progetto di dottorato, le attività svolte sono riportate di seguito. In un primo momento, è stato effettuato l'isolamento e l'identificazione molecolare degli isolati di lievito dalla raccolta 21-22. Successivamente, per superare i limiti delle metodiche cultura-dipendenti, il micobiota delle olive fermentate è stato analizzato tramite sequenziamento Illumina degli ampliconi del gene 26S rRNA. I dati molecolari sono stati inoltre complementati con la determinazione dei composti volatili effettuata con analisi Gas Cromatografia/Spettrometria di Massa (GC/MS) sia sui campioni di salamoia, che di olive.

Key words: Yeasts, spontaneous fermentation, Taggiasca olives, 26S Illumina sequencing, GC/MS.

1. Introduction

According to the PhD thesis project previously described (Traina, 2022), this poster reports the main results of the following activities:

(A2) the culture-dependent analysis (microbial viable counts) over a 6-months fermentation period with the molecular data concerning the relative abundance of fungal species;
(A3) the culture-independent analysis of the total genomic DNA (gDNA) extracted from brines and olives;

Additional results that were not included in the previous project concern the determination of volatile compounds of both brines and olives samples for batch 1 and 2.

2. Materials and Methods

Culture-dependent analysis: Taggiasca olives and brines samples deriving from two separate fermentation vessels were provided by a local company and were analyzed in duplicate from time 0 up until the end of the fermentation period (month 6). Sampling was performed at 0, 3, 7, 14, 28, 60, 90, 120, 150 and 180 days and a total of 373 yeast colonies were isolated and purified on Malt Extract agar (Generon, Modena, Italy) added with 0,5% (w/v) tetracycline (Fluka, Stainheim, Germany). For DNA extraction from isolates, 1 mL of a 24 h culture was transferred into 1,5 mL sterile microtubes (Resnova, Italy), filled with silica beads (VWR, Italy), and it was centrifuged at 13,000 x g for 5 min to pellet the cells. DNA extraction was carried out with the phenol:chloroform:isoamyl method according to Cocolin et al., 2004, and rep-PCR was carried out according to Versalovic et al., 1994, with the following modifications: initial denaturation at 95◦C for 1 min; 30 cycles at 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s; and a final extension at 72 °C for 7 min. Dendrograms generation was performed with BioNumerics ver. 6.1 software (Applied Maths, Sint- Martens-Latem, Belgium) with the Dice coefficient and unweighted pair group method with arithmetic mean (UPGMA) for hierarchical cluster analysis. Isolates with similarity percentage > 85 % were considered to belong to the same cluster and one representative from each group was subjected to amplification of the D1/D2 loop of 26S rRNA gene with primer pairs NL1-NL4, and sequenced (GENEWIZ, Germany). The sequences obtained were compared with those on NCBI website, with Nucleotide BLAST search tool. *Culture-independent analysis:* Genomic DNA was extracted from brines and olives with the MasterPure complete DNA and RNA purification kit (Epicentre, Madison, WI) and it was used for the amplification of the D1 domain of the 26S rRNA gene with primer pair NL4R (5′-GGT CCG TGT TTC AAG ACG G-3′) and LS2-MF (5′-GAG TCG AGT TGT TTG GGA AT-3′), as described by Mota-Gutierrez et al., 2019. For data analysis, sequence adapters and primers were trimmed with *cutadapter* and DADA2 algorithm was used to filter raw reads. Classification of reads was performed using the DADA2 v.1.18 pipeline, and the Silva database

was used for 26S taxonomy assignment.

3. Results and Discussion

3.1 Culture-dependent results: abundance of yeast species

The most dominant species overall were *Candida diddiensae* (36,02%), *Wickerhamomyces anomalus* (29,08%), *Aureobasidium pullulans* (13,87%) and *Pichia membranifaciens* (11,19%). In batch 1 the main species throughout the entire process were *W. anomalus* (46,19%), *C. diddiensae* (35,24%) and *A. pullulans* (6,67%), whereas in batch 2 the main representatives were *C. diddiensae* (33,74%), *A. pullulans* (26,38%) and *P. membranifaciens* (24,54%) (Figure 1).

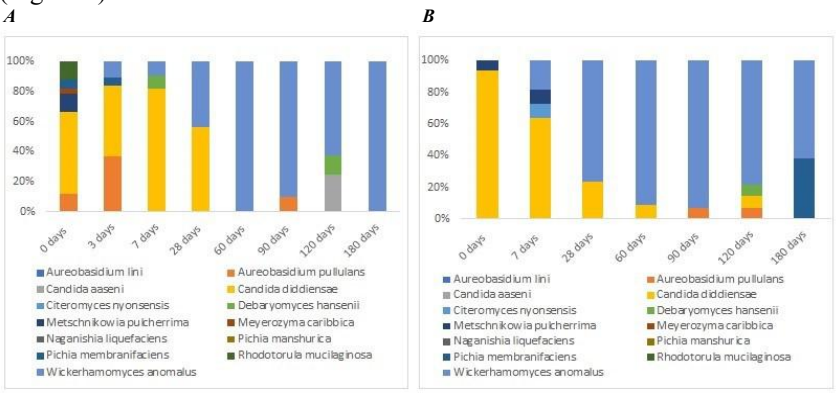
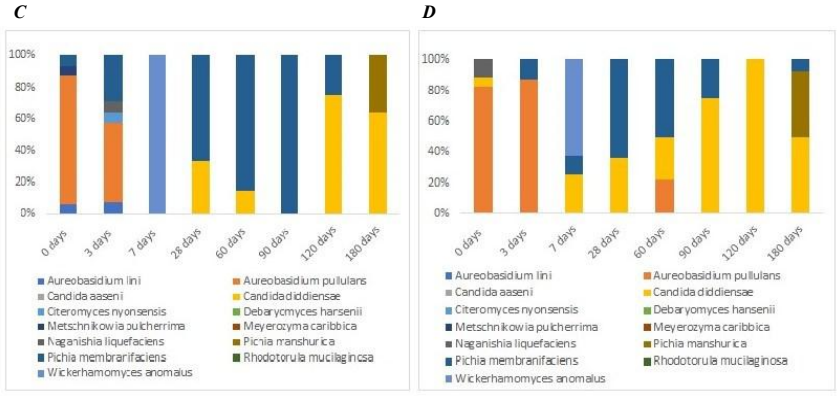


Figure 1:Relative abundances (%) of yeast species in olives (A) and brines (B) samples of batch 1 and olives (C) and brines (D) samples of batch 2. Data of brine at time = 3 days is not present because the sample was lost during transportation.

Because of the persistence of yeasts throughout the entire fermentation process (3,34 and 5 Log CFU/g in olives and brines, respectively, by the end of fermentation), confirmed by yeasts counts, and the absence of lactic acid bacteria colonies on plate (<1 CFU/g in all sampling times which represents the detection limit), we can assess that this spontaneous fermentation was mainly driven by yeast populations. The main species identified were also in agreement with (Bevilacqua et al., 2012).



3.2 Culture-independent analysis

26S rRNA gene sequencing showed a prevalence of *Aureobasidium* spp. (45,62%), *Citeromyces nyonsensis* (16,28%) and *W. anomalus* (13,05%), overall. As for batch 1, the main species found were *A. pullulans* (35,21%) and *W. anomalus* (42,99%) in olives and brines, respectively. In batch 2, the main representative species were *A. pullulans* (30,84%) and *C. nyonsensis* (67,33%) for olives and brines, respectively.

Comparing the two approaches, many differences were found in terms of detected populations in brines and olives. Correlation with GC/MS data showed that the main ASV fraction, *A. pullulans* was positively correlated to heptanal, 1-hexanol-2-ethyl and ethanol ($P < 0.05$) and negatively correlated to 1-decanol-2-hexyl in olives ($P < 0.05$).

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From Waste to Value: Unlocking the potential of red wine pomace and microalgae through tailored fermentation

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The first three activities of the PhD thesis project are described. Firstly, fermentation set-up of wine pomace and *Chlorella vulgaris* was optimized. Secondly, microbiological analysis, sugars, organic acids, ethanol, total protein, and peptides in fermented formulations were quantified. Thirdly, functional characterization of fermented substrates in terms of phenolic compounds, total free amino acids, and peptidomics profiling were demonstrated.

Dagli scarti al valore aggiunto: sfruttare il potenziale della vinaccia e delle microalghe attraverso la fermentazione guidata

Le prime 3 attività del progetto di tesi di dottorato sono di seguito descritte. In primo luogo, sono stati ottimizzati i parametri di fermentazione della vinaccia e di *Chlorella vulgaris*. In secondo luogo, le analisi hanno quantificato gli zuccheri, acidi organici, etanolo, proteine e peptidi totali dei campioni fermentati. Infine, sono state dimostrate le caratteristiche funzionali dei substrati fermentati considerando i composti fenolici, il profilo degli amminoacidi liberi totali e individuali e il profilo peptidico.

Key words: Microalgae, by-product, wine pomace, fermentation, phenolic compounds, peptidomics.

1. Introduction

In accordance with the PhD thesis project previously described, this poster reports the main results of the first three activities concerning:
(A1) optimization of fermentation set-up of wine pomace and chlorella;
(A2) microbiological analysis and assessment of sugars, organic acids, ethanol, total protein, total peptides;
(A3) investigation of phenolic compounds, total free amino acids and peptidomics profile.

2. Materials and Methods

(A1) *Chlorella vulgaris* powder and red wine pomace were chosen as starting substrates, whereas ten strains of lactic acid bacteria (LAB), including one fructophilic LAB (FLAB) and yeasts were selected for tailored fermentation. Three different mixtures were produced: *Chlorella vulgaris* (30:70), wine pomace (30:70) and a mixture of both (15:15:70). All mixtures were sterilized at 121° C for 15 min. Each mixture was then inoculated with single selected strains at an initial cell density of ca. 7 Log CFU mL^{-1} for LAB and 5 Log CFU mL^{-1} for yeasts. The samples were then incubated at 30° C for 72 hours. Raw sterile sample of each combination (stored at -20° C) and unstarted samples were considered as controls for each mixture. (A2) Microbiological analysis were evaluated and plated on agar media of de Man Rogosa and Sharpe agar (MRS), for LAB, Fructose Yeasts Extract Polypeptone agar (FYP) for FLAB, and Malt extract agar (MEA) for yeasts. Additionally, the pH of each sample was measured using a Foodtrode electrode (Hamilton, Bonaduy, Switzerland). Organic acids, sugars and ethanol were determined by High Performance Liquid Chromatography (HPLC), equipped with an Aminex HPX-87H column (ion exclusion, Biorad), a UV detector operating at 210 nm, and a Perkin Elmer 200a as the refractive index detector. Total protein quantification and total peptides quantification were determined using Bradford and o-phthalaldehyde (Opa) assays. (A3) Phenolic compounds were identified and quantified with targeted LC-MS/MS analysis using an UHPLC Dionex 3000 (Thermo Fisher Scientific, Germany) equipped with a Waters Acquity HSS T3 column (1.8 µm, 100 mm × 2.1 mm) (Milford, MA, USA) and coupled to a TSQ Quantum™ Access MAX Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific, Germany) with an electrospray source. Total free amino acids were examined with the Biochrom 30 Amino acid analyzer equipped with a NA-cation exchange column measuring 20 by 0.46 cm in inner diameter. Peptides profile of the samples were investigated with ultra-high performance liquid chromatography/high-resolution tandem mass spectrometry.

3. Results and Discussion

3.1 Determination of the microbiological and physico-chemical properties

(A2) Focusing on the interaction between protein and phenolics, we target our attention on the combination of chlorella and pomace. Preliminary analyses were conducted to characterize the substrates and the results were consistent with the available literature. All LAB and yeasts strain demonstrated their capability to grow in the

substrates, while LAB also showed their ability for acidification. HPLC analysis confirmed the conversion of available sugars into ethanol by yeasts and organic acids by LAB, with the exception of the synthesis of mannitol, which was observed only by *Ap. kunkeei* (BEE4) and *Leuc. mesenteroides* (S3d1) due to their specific metabolic pathways. Total protein content together with total peptides trend showed a common reduction during fermentation in different proportions among all the strains.

(A3) LC-ESI-MS/MS analysis enabled the identification of 17 phenolic compound in all samples (Figure 1). Most of these compounds derive from pomace, whereas in chlorella only 4-hydroxybenzaldehyde was detected. Different trends were observed during fermentation when compared to non-fermented samples. Phenolics such as 4-hydroxybenzaldehyde, caftaric acid, hyperoside, *p*-coumaric acid, procyanidin B1, procyanidin B2 and petunidin 3-glucoside have been utilized by all the strains. The reduction of polyphenols is often associated to an increase of their bioavailability, thanks to the enzymatic activity of different microorganisms that modify their complex structure and potentially enhance their absorption in human body (Gibson et al.; 2006). On the contrary, isoquercetin, isorhamnetin, myricetin and quercetins seems to be enhanced significantly during fermentation. The increase of polyphenols is associated to improve sensory feature, anti-inflammatory and antioxidant properties (Zhu et al., 2022).The total and free amino acids were presented in the heat map (Figure 2). Among the amino acids analyzed, alanine was found to be the most abundant. Proline and asparagine showed an increase after fermentation with *Lactiplantibacillus. plantarum* AFI5 and *S. cerevisiae* KFAY3, while glutamic acid was degraded by these same strains. While on the one hand increase of the total free amino acids showed to improve nutritional profile and to be potential for the formation of bioactive compounds such as bioactive peptides, on the other their decrease is often associated to reduce the bitterness or astringency and to improve the texture of fermented foods. Notably, ornithine was only detected in the fermented samples with *Weissella cibaria* P9 and *Lc. Lactis* WSL2. UHPLC/HR-MS/MS investigated the peptide profile of the samples (data not shown). The screening of peptides with a molecular weight lower than 100 kDa identified a total of 7708 distinct peptides across the 14 analyzed samples. After fermentation, an overall increase in peptide levels was observed by all strains, especially by *W. cibaria* and *Leuc. Mesenteroides* (739 and 645 additional peptides, respectively). Furthermore, the length of the peptides was evaluated, focusing on lengths ranging from 6 to 11 amino acids, the most inclined to have bioactivity properties. In terms of total peptides, *Lb. plantarum* had the lowest amount within this range, with 928 peptides, followed by *Ap. Kunkeei* with 933 peptides. The highest amount was found in Spontaneous, *W. cibaria*, and *S. cerevisiae*, with 1043, 1047, and 1045 peptides, respectively. All 7708 identified peptides underwent cross verification and validation using the BIOPEP-UWM database (Minkiewicz et al., 2019), which comprises a comprehensive list of 4,670 known bioactive peptides. Unfortunately, none of the sequence of the identified peptides shared 100% homology in the amino acid sequence, with previously identified bioactive peptides indicating the absence of known bioactive peptides in the sample. However, the software utilized for this analysis is not able to detect the peptides of lee than 5 amino acids, leaving margin for further research. Further analysis will deal with the selection of the best-performing starters and the samples will be further characterized by bioactivity *in vitro* analyses.

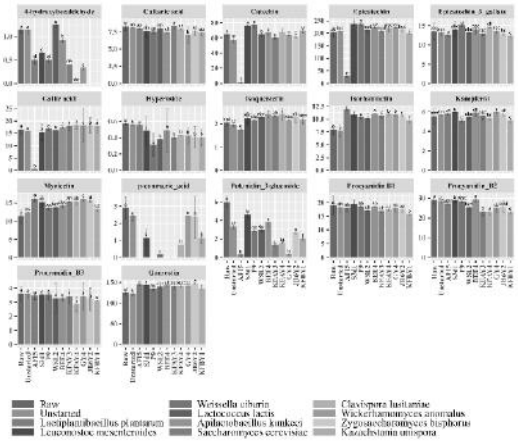

Figure 1 *Quantification of phenolic compounds (mg L^{-1} FW) by LC-ESI-MS/MS among all the tested samples.*

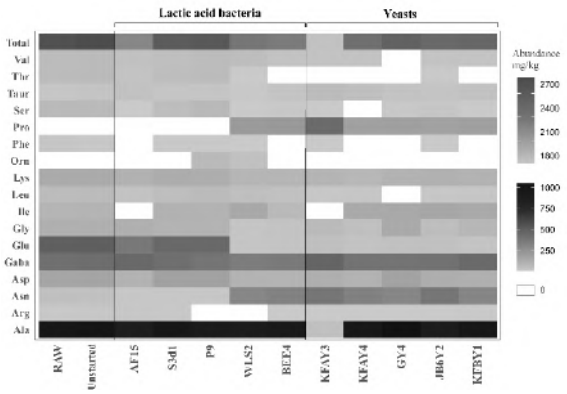

Figure 2 *Abundance of total free amino acids(mg kg-1) by Biochrom 30 Amino acid analyzer among all the tested samples.*

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Green sensors and smart services for the optimization of agri-food supply chains with a view to industry 4.0: greater sustainability of production, business competitiveness and reduction of food waste

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The PhD project is aimed at design a portable, user-friendly, and low-cost hyperspectral (HSI) sensor that will be applied on fresh-cut products in laboratory conditions to evaluate some quality parameters. The hyperspectral prototype underwent a first calibration phase in which specific light sources were used (i.e., halogen lamp and LEDs). Secondly, the performance of the HSI camera was evaluated in controlled conditions. A first test was performed on a standard (i.e., Rubik's cube) and then on food matrix.

Sensori green e servizi intelligenti per l'ottimizzazione delle filiere agroalimentari in un'ottica di industria 4.0: maggiore sostenibilità della produzione, competitività delle imprese e riduzione degli sprechi alimentari

Questo progetto di dottorato ha lo scopo di sviluppare un sensore iperspettrale portatile, facile da usare e a basso costo che verrà utilizzato in condizioni di laboratorio su prodotti di IV gamma per valutarne alcuni parametri qualitativi. Il prototipo iperspettrale ha subito una prima fase di calibrazione in cui sono state utilizzate specifiche sorgenti luminose (i.e., lampada alogena e LED). In un secondo momento, le performance della telecamera iperspettrale sono state valutate utilizzando un set-up di acquisizione in condizioni controllate. Un primo test è stato eseguito utilizzando uno standard (cubo di Rubik) e successivamente una matrice alimentare.

Keywords: Hyperspectral imaging, cost-effective sensor, image processing, food quality, post-harvest.

1. Introduction

In accordance with the PhD thesis project previously described (Vignati, 2022), this poster reports the main results of the first activities concerning:

(A1) the testing phase in laboratory conditions of the hyperspectral (HSI) camera prototype, in terms of calibration of the device and application of hyperspectral imaging on food matrix, thanks to the development of a software for image acquisition and elaboration in MATLAB® environment;

(A2) the application of chemometrics techniques using MATLAB® to analyse the hyperspectral data obtained.

2. Materials and Methods

Firstly, the assembled HSI prototype has been calibrated by positioning the device in an acquisition stage in order to acquire pictures of light sources which emit at predefined wavelengths (i.e., red, green and blue LEDs, and a halogen lamp). Such a critical process allows to identify the spatial position of the pixels corresponding to the wavelengths of the electromagnetic spectrum. Thus, five calibration images have been acquired:

- The first image was acquired to identify the Region of Interest (ROI) corresponding to the real image, using a halogen lamp (darkroom not needed in this case).
- The second image was acquired using the halogen lamp inside the darkroom in order to identify extension of the diffracted spectrum (first order of diffraction) from the visible (Vis) to the Near Infra-Red (NIR) spectral regions.
- The third, fourth and fifth are acquired inside the darkroom for each LED, in the Red (R), Green (G) and Blue (B) channels to identify each peak of emission with a well-known wavelength inside the visible range of the spectrum. (Salazar-Vazquez and Mendez-Vazquez, 2020).

Each emission peaks of the RGB channels were determined using a portable spectrophotometer.
Once calibrated, the performances of the prototype were evaluated using a specific set-up in order to standardize the image acquisition in lab-scale conditions. The set-up was composed by an integrating sphere (hollow hemisphere with perfectly diffusing internal surface that allows a complete reflection of the light toward the sample), a camera holder and light bulbs (halogen lamps and LEDs) inside the sphere and that can cover a spectral range from 300 to 1000 nm. The HSI camera was positioned at 70 cm from the sample, and a first test was performed using a common 3x3 Rubik's cube (with a line of red squares, a line of blue ones and one of green) to obtain a linear scan of the image and use it to visually evaluate the accuracy of the hyperspectral device. Then, tests using a food sample were performed in order to have an initial screening of the capability of the system to detect spectral differences in the food sample.

3. Results and Discussion

All the acquired images (both of calibration and the sample ones) are composed of three parts (Fig. 1): in the middle there is the real image (i.e., the light source or the sample) or ROI, while on its sides there is the diffracted light (called "first order of diffraction"); the axes represent the chosen resolution of the camera (1280x720).
As mentioned, the calibration images were elaborated with MATLAB® in order to: (i) determine the area of the central ROI and (ii) the length of the first order of diffraction corresponding to the spectral range of 400-1000 nm, and (iii) to identify the centroids of each RGB channel based on the ROI area (Fig. 2 – on the left). Finally, at each centroid was assigned the corresponding value of the emission peak of the LED, i.e., 632 nm, 522 nm and 462 nm for R, G, and B, respectively (Fig. 2 – on the right).

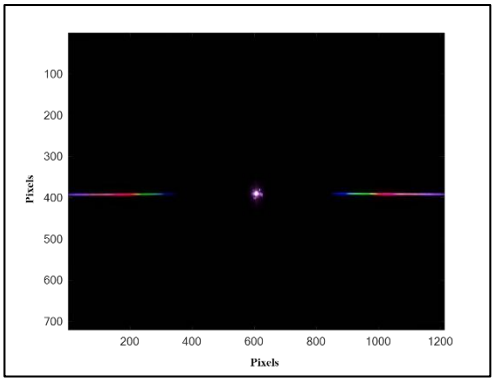

Figure 1 *Calibration image acquired inside the darkroom with a halogen lamp. It is composed by three parts: the ROI or real image in the centre, the first order of diffraction on the left and the first order of diffraction on the right.*

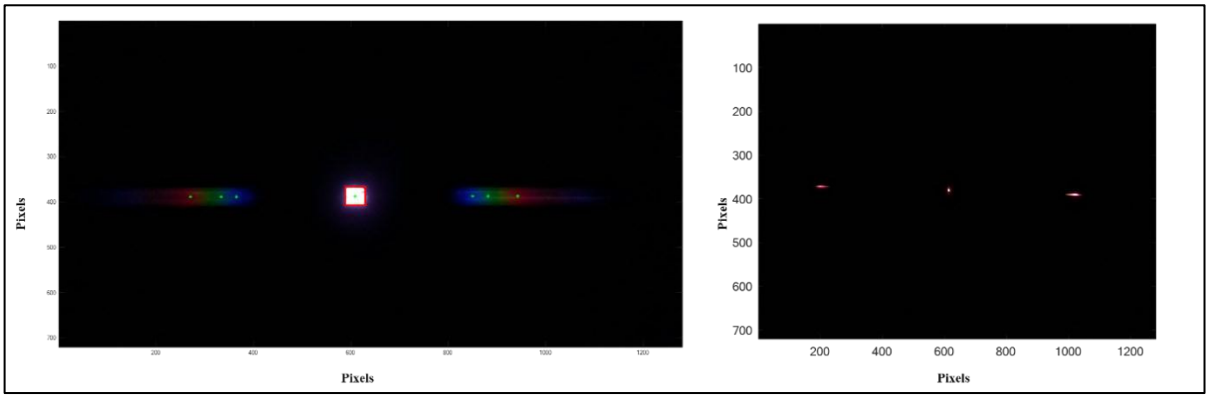

Figure 2 *ROI (in red) and centroids (in green) identification in the image acquired outside the darkroom with a halogen lamp (on the left) and the image acquired inside the darkroom with the Red LED (on the right).*

The first test was performed using a Rubik's cube as a standard in order to compare the spectrum of each coloured face of the cube with the spectrum extrapolated from the diffracted image. Then, tests using a food matrix were performed using firstly an apple with a damaged tissue to have a first screening of the capability of the system to detect spectral differences between the sound and the damaged tissues (Fig. 3). Some differences between the two types of tissues were identified, such as the shape, intensity and presence/absence of peaks at specific wavelengths (e.g., the sound tissue has a higher peak at 630 nm, and there is a slight signal in the blue region at about 522 nm that is absent in the case of the damaged tissue). The experimental results agree with the expected ones and further tests on other food matrix are still ongoing. Thus, the PhD thesis project can proceed without any significant modification.

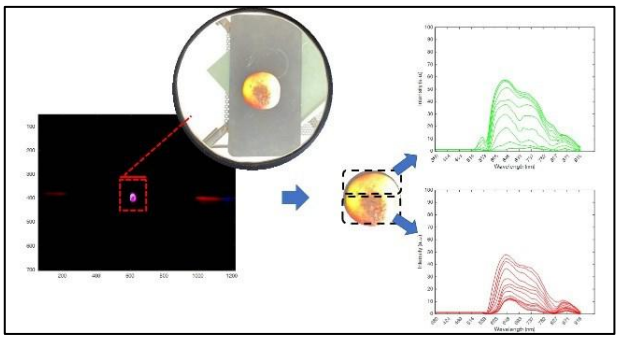

Figure 3 *Image acquired on an apple (on the left) and the corresponding spectra of the sound tissue (in green) and of the damaged tissue (in red).*

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Acceptability And Nutritional, Metabolic, Functional Impact Of An Italian-Mediterranean, Sustainable, Plant-Based Dietary Pattern

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Activities carried out during the first two years of the PhD are related to a pilot dietary intervention study aiming to evaluate the effects of a plant-based diet, called EAT-IT. Participants metabolic and functional markers were analysed to assess health-related outcomes. Being a critical point of these dietary patterns, the acceptability was evaluated; in addition, food intake, consumption of ultra-processed foods and adherence to Mediterranean diet was evaluated to understand the characteristics of the dietary patters. These results will help to find out new strategies to facilitate and increase the adoption of plant-based diets for the Italian population.

Accettabilità e impatto nutrizionale, metabolico e funzionale di un modello alimentare italo-mediterraneo, sostenibile, vegetale

Durante i primi due anni del dottorato, le attività svolte sono state relative a uno studio pilota di intervento dietetico volto a studiare gli effetti di una dieta plant-based, chiamata EAT-IT. L'effetto sullo stato di salute è stato analizzato tramite la valutazione di marcatori metabolici e funzionali dei partecipanti mentre la determinazione delle caratteristiche del pattern alimentare è stata ottenuta attraverso l'analisi dell'assunzione dietetica, del consumo di alimenti ultra-processati e dell'aderenza alla dieta mediterranea; infine, è stata valutata l'accettabilità essendo questa un punto critico di tali modelli dietetici. Questi risultati aiuteranno a trovare nuove strategie per facilitare la popolazione italiana ad aumentare l'adozione di diete plant-based.

Key words: Dietary patterns; Sustainability; Health-related markers

1. Introduction

In accordance with the PhD thesis project previously described (Vinelli, 2021), this poster reports the main results of the first activities concerning:
(A1) analysis of nutritional, metabolic, functional parameters from biological samples collection;
(A2) evaluation of dietary intake and nutritional characteristics of the EAT-IT dietary pattern.

2. Materials and Methods

2.1 Study design

A single-blind, randomized controlled, crossover trial was performed. Subjects eligible to be enrolled had to be healthy, aged >18 years old and not following a specific diet (e.g. vegetarian or vegan diet). Participants were randomly allocated to follow the EAT-IT diet or an IDG-based control diet; diets were personalized based on individual energy and nutrient requirements. The two phases were separated by a washout period and the three phases lasted 6 weeks each. Before and after each intervention, weighted food records and biological samples were collected, such as blood, fecal and urine samples to assess health-related parameters.

2.2 Nutritional assessment

During each intervention, nutrient intake was assessed through 7-day weighted food records, while acceptability, NOVA ultra-processed foods consumption, level of adherence to the Mediterranean Diet were determined through validated questionnaires.

2.3 Biomarkers

Biochemical biomarkers assessed were: creatinine Jaffe (mg/dl); uric acid (mg/dl); AST/GOT (U/L); ALT/GPT (U/L); GGT (U/L); triglycerides (mg/dl); fasting glycemia (mg/dl); C-reactive protein hs (mg/L); cholesterol (mg/dl); HDL (mg/dl); LDL (mg/dl); insulin (uU/ml). All these parameters were determined using standard laboratory methods. In addition, serum samples will be used for future analysis in estimating dietary exposure to substances present in food like chemicals and contaminants, intentionally added or naturally present.

2.4 Gut microbiota composition and SCFAs

Fecal samples were used to analyze gut microbiota composition by 16SrRNA gene quantification and taxonomic profiling. SCFAs, in particular lactic, acetic, succinic, propionic, butyric, isobutyric, isovaleric, valeric acid were evaluated by ultrahigh-performance liquid chromatographic-Orbitrap mass spectrometry (LC-HR-MS).

2.5 Inflammatory parameters and DNA damage

Serum TNF-alfa, adiponectin, IL-6, LBP and endothelin were evaluated by enzyme-linked immunosorbent assay (ELISA) kits. DNA damage was assessed by Comet Assay through an enzymatic treatment with FPG.

2.6 Statistical analysis
SPSS software was used to evaluate the significance of the results obtained.

3 Results and Discussion

Baseline characteristics of the 9 subjects who completed the study were as follow. Age: 25 ± 2 (23 to 30 years); gender (n, %): female 5 (50), male 4 (40); education (n, %): high education (university level) 10 (100); n household component 2.1 ± 1.2; height (m): 1.7 ± 0.1; BMI 21.4 ± 1.5.

3.1 Nutritional assessment
The evaluation of dietary intake showed no differences in total energy and other nutrients intake despite a statistically significant increase of vegetal protein/total protein ($p<0.001$) and dietary fibers ($p<0.001$) intake compared to the control group.
Regarding the acceptability, it was reported "sufficiently good" and tolerable in terms of preparation (not easy, but not difficult), only one subject evaluated the diet as "extremely unappealing" and "extremely difficult" to be prepared. On average, the habitual diet has been judged easier than the EAT-IT diet which was considered quite acceptable and potentially feasible in the long-term. In conclusion, the diet was easily accepted and consumed on average except for the preparation of some products such as legumes. In this context, useful strategies and recipes are necessary to help and facilitate the adoption of plant-based diets and will be integrated in a more acceptable and feasible Mediterranean healthy and sustainable dietary pattern.
Furthermore, the consumption of ultra-processed foods has been assessed with NFFQ while the level of adherence to Mediterranean diet by MEDI-LITE questionnaire; both questionnaires showed not differences between EAT-IT and IDG diets.

3.2 Biomarkers
Among biochemical parameters, a statistically significant decrease of insulin (8,44 ± 2,18; 6,53 ± 2,24 uU/ml; $p<0.05$) and a reduction of cholesterol (181,2 ± 42,5; 166,1 ± 39,4 mg/dl) close to significance ($p=0.067$) were found after the EAT-IT pattern intervention.

3.3 Gut microbiota composition and SCFAs
In addition, gut microbiota composition and total SCFAs did not change overtime following the EAT-IT diet probably due to the high inter-individual variability amongst the participants and the small sample size. However, a positive correlation between total fibre intake and valeric and isovaleric acid was found. Furthermore, gut microbiota β-diversity resulted very variable and was greater between subjects than within the same subject as reported in figure 1.

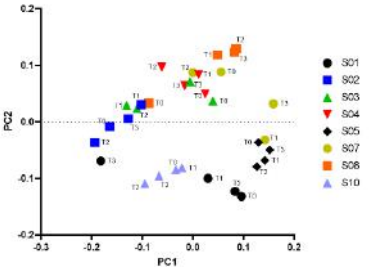

Figure 1 *Principal coordinates analysis of weighted UniFrac distance*

3.4 Inflammatory parameters and DNA damage
Serum TNF-alfa, adiponectin, IL-6, LBP and endothelin levels were evaluated by ELISA kits.
No significant effect of treatment, nor treatment x time interaction was found between the EAT-IT and IDG for adiponectin and endothelin; results from other markers are still under elaboration.

This pilot study represents the first step on the pathway towards the optimization of the EAT-IT diet to a more feasible pattern improving adherence to an Italian-Mediterranean diet. In fact, it is important to underline that the EAT-IT recommendations were quite far from the current dietary habits of the Italian population in terms of portion sizes and frequencies of consumption, especially for legumes and nuts. The results of this study showed difficulty in preparing legumes-based meals. For this reason, future steps will focus on elaborating strategies able to facilitate the preparations of plant-based meals, such as choosing different food sources and increasing different cooking methods that could also potentially impact on the final nutrient intake. Future analysis on health-related parameters will help exploring the potential beneficial effects of optimized diets in different target of populations.

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Flat breads: past, present and future

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The initial activities of this PhD project have been focused on the study of the flat breads typical of the Mediterranean basin and then the innovation of selected products with legume protein. Preliminarily, a survey was conducted by collecting information in a database of all flat breads of the nine countries involved in the FlatBreadMine Project. Subsequently, a gluten-free *focaccia* has been formulated, with the design of experiments approach, to study the effect of the dry-fractionated pea protein, rice and corn flour on the physicochemical and sensory properties, to obtain a product with a good texture, organoleptic and nutritional profile.

Pani piatti: passato, presente e futuro

Le prime due attività del progetto di dottorato hanno riguardato lo studio dei pani piatti tipici del bacino del Mediterraneo e l'innovazione di prodotti selezionati con proteine dei legumi. Preliminarmente, è stata condotta un'indagine per raccogliere in un database le informazioni di tutti i pani piatti dei nove Paesi coinvolti nel progetto FlatBreadMine. Successivamente, è stata formulata una *focaccia* senza glutine, con l'approccio del disegno sperimentale, per studiare l'effetto delle proteine di piselli dry-fractionated, farina di riso e mais sulle proprietà fisico-chimiche e sensoriali, per ottenere un prodotto con una buona consistenza, profilo organolettico e nutrizionale.

Key words: Flat bread; Mediterranean basin; *focaccia*; legume protein; dry fractionation; mixture design.

1. Introduction

In accordance with the PhD thesis project, this poster reports the main results of the first two activities concerning:
(A1) the valorization of the flat bread typical of Croatia, Egypt, France, Greece, Italy, Jordan, Lebanon, Malta and Spain, involved in the FlatBreadMine Project, and the analysis of the technical and cultural features;
(A2) the innovation of gluten-free *focaccia*, formulated with dry-fractionated pea protein, to study how the different ratios of ingredients influenced the quality of flat breads.

2. Materials and Methods

The information on flat breads to include in the database has been collected as reported in Pasqualone et al (2022). To formulate a gluten-free *focaccia*, preliminary trials were carried out to define the experimental domain. A simplex-centroid mixture design was planned, considering rice flour (RF) ($15 \leq RF \leq 30$ g); corn flour (CF) ($15 \leq CF \leq 30$ g); dry-fractionated pea protein at 55% of protein content (PP) ($0 \leq PP \leq 15$ g) (Table 1). The sum of the components was 45 g/100 g, whereas the other 55 g/100 g were constituted by water (50 g), yeast (1 g), salt (1.5 g), psyllium husk powder (2.5 g) and were kept constant. The image analysis of the *focaccia* was carried out with the procedure described by De Angelis et al. (2023). Three replicates were carried out. The firmness was evaluated by a texture profile analysis (TPA), according to Pasqualone et al. (2019). Four replicates were carried out. The typical odor associated with legumes and corn was evaluated by a trained sensory panel of eleven people (5 male, 6 females, age 23-55 y) and the intensity was scored on an anchored 0 - 9 scale (not perceived - highly perceived). The optimal formulation has been identified considering the global results. Then, the nutritional composition of the optimized *focaccia* has been evaluated, according to the AOAC Official methods, conducting the analyses in triplicate. The responses were modelled according to the postulated special cubic model and the regression coefficients (R^2), the adjusted coefficients of determination (R^2 adj), as well as their significance ($p \leq 0.05$) were calculated by the software Design-Expert 11 (StatEase Inc., Minneapolis, USA).

3. Results and Discussion

3.1 Flat bread database

The information on flat breads has been included in an online available database (https://flatbreadmine.eu/resources/) reporting, for each flat bread, the original area, the diffusion, the ingredients, the raw material characteristics, the type of yeast, the additional ingredients, the production process, the characteristics of bread, and the sources of information. The database is organized in an Excel file and has 27 columns and 143 rows in which are catalogued 51 single-layered flat breads, 15 double-layered, 66 garnished and 11 fried (Pasqualone *et al*., 2022). Italy has a large number of products, principally garnished, which often are recognized with Quality Marks (70 out of 91). In particular, three are Protected Geographical Indications (*Piadina*

Romagnola; Schuttelbrot; Focaccia di Recco), one is a Traditional Specialty Guaranteed and Intangible Heritage of Humanity (UNESCO) (*Neapolitan pizza*), one is Slow Food Presidium (*Testarolo Pontremolese*), sixty-three are Traditional Agri-Food Products (PAT) and two are Municipal Denominations (De.Co.) (*Crostolo di Urbania; Farinata di Imperia*). Finally, seven flat breads are in the Slow Food Ark of Taste, an expression of tradition and endangered history (Pasqualone *et al*., 2022). Considering the ingredients, wheat flour is the most used, but also other cereals, legumes and chestnut flour were recorded (Pasqualone *et al*., 2022). The most used leavening agent is baker's yeast, however, about 9% of the breads are formulated with chemical yeast and about 20% are unleavened. An interesting aspect to consider is baking: sixteen different traditional baking techniques have been surveyed, some of which have very ancient origins (Pasqualone *et al*., 2022).

3.2 Optimized gluten-free *focaccia* formulation

The properties of *focaccia* varied among the seven trials, being significantly affected by the ratio of the ingredients, as reported by other authors in gluten-free bread (Ziobro *et al*., 2016). The crumb of the flat breads prepared with PP was characterized by the presence of numerous cells of small dimensions (Figs. 1a and 1b). The results suggested that the increasing percentage of PP increases the firmness of bread (Fig. 1c).

Table 1 *Formulation of the samples according to the simplex-centroid mixture design. RF = rice flour; CF = corn flour; PP = pea protein. *Replicates*

Sample	RF	CF	PP
1	30	15	0
2	20	20	5
3	15	15	15
4	22.5	15	7.5
5*	15	15	15
6*	30	15	0
7	22.5	22.5	0
8	15	30	0
9	15	22.5	7.5
10*	15	30	0

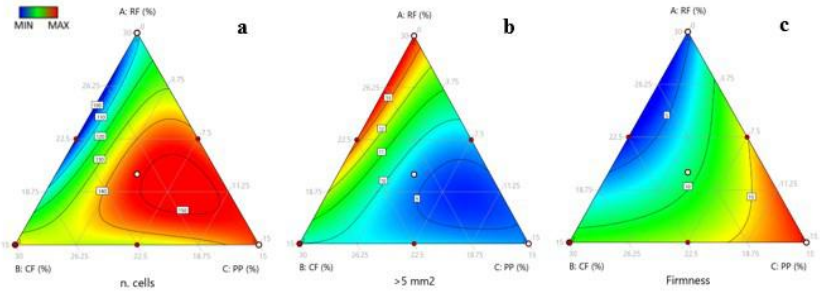

Figure 1 *Contour plots of the number of cells (a); percentage of cells > 5 mm² (b); firmness (c) of the crumb of flat breads.*

Fig. 2 shows the sensory properties (legume odor and corn odor) of the gluten-free *focaccias*. As attended, the higher percentage of PP increased the perception of legumes in the *focaccia* sensory profile, while the odor of corn was perceivable but partly masked by the legume odor brought by PP. *Focaccia* added of 5% PP was identified as optimal, considering the balancing of nutritional aims and the textural and sensory features. The *focaccia* presented 40.53±1.21% of moisture, 8.27±0.34% of proteins, 0.73±0.01% of lipids, 50.48±1.37% of carbohydrates, 3.92±0.22% of fibers and 233.7±5.0 kcal/100 g. This optimized *focaccia* fulfilled the EC Reg. 1924/06 for the nutritional claims "source of protein" (>12% of the energy value provided by proteins), "source of fiber" (>3 g/100 g) and "low-fat" (<3 g/ 100 g).

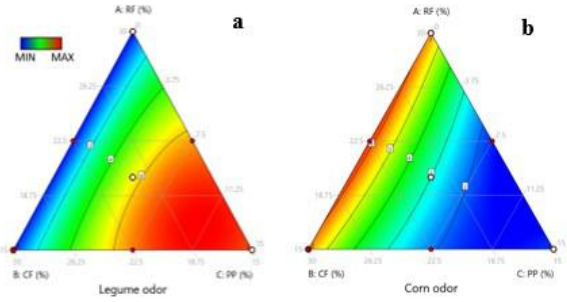

Figure 2 *Contour plots of legume (a) and corn odor(b)*

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Regulation (EC) No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods (2006) *Off J Eur Union* **404**: 9-25.

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Workshop contributions

3st year - PhD Oral Communications

Valorisation of South Tyrolean Food Products Through the Study of Their Antioxidant Behaviour

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This PhD thesis dealt with the assessment of the antioxidant behaviour of different food products typical in South Tyrol. The antioxidant behaviour depends on both the antioxidant activity and capacity of a matrix, nevertheless its analytical composition. A novel DPPH• kinetic model was developed to precisely study the antioxidant activity and capacity of analytical standards and was then validated on food extracts. Further analyses with a coulometric detector (CAD) were employed to investigate which molecules in the matrix were responsible for the antioxidant properties.

Valorizzazione dei Prodotti Alimentari Altoatesini Attraverso lo Studio del Loro Comportamento Antiossidante

Questa tesi di dottorato ha trattato lo sviluppo di un modello cinetico per studiare il comportamento antiossidante di diversi prodotti alimentari tipici dell'Alto Adige. Il comportamento antiossidante dipende sia dall'attività sia dalla capacità antiossidante di una matrice e dalla sua composizione analitica. È stato sviluppato un innovativo modello cinetico basato sul DPPH• per studiare l'attività e capacità antiossidante di standard analitici prima e di estratti alimentari dopo. Sono poi state eseguite successive analisi con detector coulometrico (CAD) per determinare quali molecole presenti in un estratto avesse effettivamente proprietà antiossidanti.

Key words: DPPH•; kinetics; antioxidant activity; LC-MS, apples, herbs, fruit juices.

1. Introduction

In accordance with the PhD thesis project previously described (Angeli, 2022), this oral communication reports the main results of the following three activities directed to:

A1) develop a simple but efficient kinetic model based on the DPPH• radicals that involved not only the main reaction described in literature, but also a side reaction whose presence was demonstrated with mass spectrometry. The model was applied on officinal herbal extracts.

A2) optimize the developed kinetic model for very fast antioxidants, i.e., ascorbic acid, whose reaction ended within 2 s, with a stopped-flow apparatus and apply it on fruit juices.

A3) study the antioxidant activity and capacity of three different apple varieties with the developed model; investigate the antioxidant composition of the extracts with the triple detector (LC-UV-MS-CAD) to determine which molecules were responsible for the different behaviour of the apples.

2. What Do South Tyrolean Food Products Have in Common?

South Tyrol is a region in the North of Italy, where the Italian mixes with the Austrian culinary tradition. The alpine environment encourages the cultivation of officinal herbs at high altitudes, as well as apples and other fruits (Ceci et al. 2021). These food products that are major ingredients in the South-Tyrolean kitchen share the high content in micronutrients, such as vitamins and phenolic compounds. These constituents are important not only for human health, but also to preserve the quality of a food product in terms of antioxidant properties (Ding et al. 2022).

3. DPPH• Kinetics Vs the Conventional Spectrophotometric Assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH•) is a stable free radical that is commonly used to characterize the antioxidant capacity of methanolic extracts in the so-called DPPH• assay. DPPH• is widely used thanks to the velocity of the reaction, the facility in the measurement and the stability of the radical. The conventional protocol is based on the single-point measurement of the absorbance of DPPH• at its maximum (usually 515 nm) after 30 min or 1 h reaction. A limitation of the common assays is that they fail to provide temporal information that can be used to distinguish the rate at which different antioxidants produce their antioxidant effect (Foti et al. 2004). The rate constant decreases in aprotic solvents and increases in alcohols, demonstrating that a main electron-transfer mechanism is involved rather than H-atom transfer. Several new and alternative approaches have been

revised in recent years, underlining the drawbacks of the lack of a standardized method. For this reason, this work aimed at proposing a method suitable for the analysis of antioxidant standards that could be applied also to food extracts.

3.1 The DPPH• kinetic model

The mechanism involved in the reaction between the DPPH• radical and the antioxidant molecule is described in Equation (1):

$$AH + DPPH^{\bullet} \xrightarrow{k_1} A^{\bullet} + DPPH - H \quad (1)$$

where all symbols are given in section 9.

However, the possible side reactions occurring between the transient radicals (A•) generated in the first reaction with DPPH• have not been fully considered yet. Such radicals are generally able to quench another DPPH radical, as illustrated in Eq. (2):

$$A^{\bullet} + DPPH^{\bullet} \xrightarrow{k_2} Products \quad (2)$$

This reaction is largely underestimated and could explain the variability of the results reported in the literature. Recently, Foti and colleagues accounted for this side reaction. They measured the reactivity of antioxidants with the DPPH• radical applying a kinetic model of pseudo-first (when antioxidants are in excess) or second order (when the concentration of DPPH• is equal to or higher than that of antioxidants). The authors were able to deduce the presence of side reactions graphically by following the DPPH• decay over time. However, proof of this side reaction based on a mechanistic model was still not demonstrated.

Moreover, a comprehensive model also considering the stoichiometric factor, n (Eq. 3), has not been proposed before (Angeli et al. 2021).

$$n = \frac{[DPPH_0^{\bullet}] - [DPPH^{\bullet}]}{[AH_0]} \quad (3)$$

3.2 DPPH• kinetics for fast antioxidants: optimization with a stopped flow system

Kinetic methods are difficult to be applied for studying fast antioxidants, like, for example, ascorbic acid. Despite its widespread use, the radical scavenging activity of ascorbic acid is not well characterized, likely, because its reaction with radicals, is so fast that the reaction goes to completion within a few seconds. This makes difficult to perform kinetic studies with spectroscopic measurements, i.e., performed with classical glass cuvettes. In such cases, the reduced form of ascorbic acid falls rapidly to zero even during its mixing with radicals, like DPPH•. To overcome such limitations, the kinetic-based DPPH• method could be greatly improved if implemented with a stopped-flow technique. A stopped flow apparatus is especially suitable for studying fast reactions, in which two or more reactants can be rapidly mixed, and delivered through a flow cell detector with a dead time of just a few microseconds (Angeli et al. 2023).

4. Application of the Model to South Tyrolean Food Products

One of the strengths of the kinetic model is its standardization. Since the rate constant decreases with the increase of the antioxidant concentration, the food extracts were all standardized to 30 µM of GAE. The model was successfully applied to eight herbal extracts provided by the local company Naturalsalus®, among which, *Moringa oleifera*, *Harpagophytum Procumbens*, *Turnera aphrodisiaca*, *Urtica dioica*, *Rhodiola rosea*, *Melissa officinalis*, *Fraxinus excelsior* and *Filipendula Ulmaria* (Angeli et al. 2021). The antioxidant activity of three ricotta whey vinegars produced by the local company Algunder Sennerei® was determined using the DPPH• kinetic model and discussed in a master thesis project. The model was tested also on fruit juices, helping to understand their antioxidant activity and capacity (Angeli et al. 2023). And finally, it was applied to study the antioxidant behavior in a non-browining ("Majda") and a red-flesh (Kissabel rouge) apple variety versus a control variety (Golden delicious) (Cebulj et al. 2023).

5. Who and How is Responsible for the Antioxidant Activity and Capacity? From the DPPH• Kinetics to the LC-MS-CAD

The DPPH• kinetics is a very useful tool to study the antioxidant behaviour of a food item, but the employment of more sophisticated techniques is necessary to understand which molecules in the extracts are responsible for it. Therefore, liquid chromatography coupled to mass spectrometry and the coulometric detector was used to identify and quantify the molecules showing a redox behaviour in the three apple varieties.

6. Materials and Methods

Methanol, DPPH•, Folin reagent, Na_2CO_3, and standards reagents (ascorbic acid, α-tocopherol, Trolox, catechin, epicatechin, quercetin, rutin, tannic, ellagic, 3,4-dihydroxybenzoic, and syringic acid) were all purchased from Sigma Aldrich (St. Louis, MO, USA) at the highest available grade. Phloretin with a purity higher than 98% was purchased from Tokyo Chemical Industry (Zwijndrecht, Belgium). Stock solutions of each antioxidant were prepared in methanol at a final concentration of 10 mM. Stock solutions of DPPH• were prepared in methanol at the concentration of 2.5 mM. All solutions were prepared daily.

The kinetic-based DPPH• method was performed with a stopped-flow system (RX2000, Applied Photophysics, Leatherhead, UK) equipped with a pneumatic pump, a quartz flow-cell and a Cary 60 UV–VIS spectrophotometer (Agilent Technology, Santa Clara, CA, USA). The stopped-flow system had two syringes, one loaded with 200 µM DPPH• solution, and the other with the antioxidant at concentrations between 20 and 200 µM. It should be noted that the DPPH• and antioxidant solutions were prepared at a doubled concentration than the one desired, since there is a 1:1 dilution after the mixing of the two reagents. The concentration of the food extracts was therefore standardized at 60 µM of GAE. Priming was performed before every run by flowing the two reagents in the system. As soon as the pneumatic drive was pressed, equal volumes of the two solutions were mixed and transferred into the quartz flow cell, with a max delay of 6 ms. The resulting absorbance of the reaction mixture was recorded every 18 ms, at a wavelength of 515 nm, by the UV spectrophotometer. Simulation and fitting of the reaction kinetic data were performed with the software Copasi (version 4.29).

Total phenolic content (TPC) was estimated with the Folin–Ciocalteu's reagent using the method of Singleton et al. with slight modifications17. Briefly, a volume of the juice sample (130 µL) was mixed with distilled water (1 mL) and the Folin reagent (130 µL). After 5 min, 130 µL of Na_2CO_3 solution (20%) were added. The mixture was vortexed, incubated for 2 h in the dark at 25 °C and transferred in a microplate well (UV-StarR microplate, 96 wells, Greiner Bio one, Frickenhausen, Germany). The absorbance was read at 765 nm with the spectrophotometer (Infinite M Nano+ , Tecan, Mannedorf, Switzerland). Results were expressed as mg/100 mL of gallic acid equivalents (GAE) from a calibration curve built with standard solutions of gallic acid.

HPLC couple to mass spectrometry and coulometric detector was employed for antioxidant molecules identification, as described in (Ding et al. 2022; Ding et al. 2023). The advantages of the coularray detector has been previously discussed (Razem et al. 2022).

7. Results and Discussion

7.1 Application of the DPPH• kinetic model to standard analytes

The DPPH• kinetic model was first applied to standard analytes, as reported in Table 1. Overall, from the comparison of the results obtained with or without the side reaction, it is evident that the side reaction improves the fitting of the kinetic model. Even more importantly, the side reaction can be used to obtain stoichiometric factors that are predictable from the chemical structure of the antioxidants. When the side reaction was not included in the model, the resulting k_1 was underestimated.

Table 1 *Observed rate constants, k1 and k2 (M-1s-1), R2 values and stoichiometric factors, n, for the reaction of DPPH• 100 µM with antioxidants (2–7) 10 and 100 µM at 25 °C in methanol, without and with side reaction. The values obtained are the mean of at least three repetitions, with a standard deviation of max 20%.*

Antioxidants	Results without Side Reaction			Results with Side Reaction			
	k_1 (10^3 $M^{-1}s^{-1}$)	R^2	n	k_1 (10^3 $M^{-1}s^{-1}$)	k_2 ($M^{-1}s^{-1}$)	R^2	n
2 gallic acid	0.42–0.57	0.987	4.9	1.13–0.66	145–23	0.997	2.9
3 caffeic acid	0.7–0.24	0.985	2.6	0.81–0.51	4–110	0.993	2.4
4 chlorogenic acid	0.19–0.04	0.999	2.3	0.2–0.04	0.6–8	0.999	2.1
5 ferulic acid	0.11–0.08	0.984	1.3	0.33–0.15	45–26	0.998	0.8
6 ascorbic acid	11.5–3.63	0.996	1.9	11.5–3.63	-	0.996	1.9
7 Trolox	0.54–0.39	0.999	2.2	0.54–0.39	-	0.999	2.2

7.2 Demonstration of the side reaction

The DPPH• kinetic model was validated with the finding of the unknown products of Eq. (2) with LC-HRMS for only those standards that showed the side reaction in the kinetic model. The fragmentation spectra of the products show the typical fragments of DPPH• at m/z 225.9984 and 196.0011.

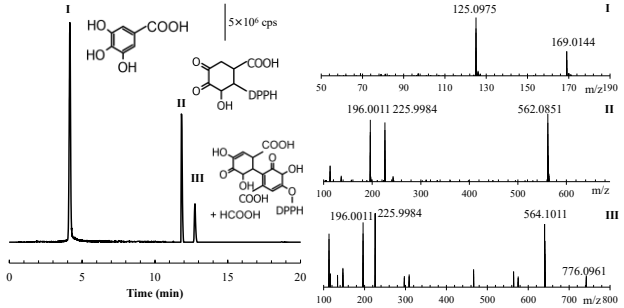

Figure 1 *Extracted ion chromatographic peaks with the corresponding proposed molecular formulas for gallic acid after the reaction with DPPH•. On the right, the dd-MS² spectra presenting the characteristic fragment ions are reported.*

7.3 Optimization of the model with the stopped flow apparatus

Figure 2 shows the transient signal of DPPH• (100 µM) after the rapid mixing with A- 10 µM of vitamin C and B - 50 µM of (a) phloretin and equimolar concentrations (10 µM) of, (b) α-tocopherol, (c) catechin, (d) epicatechin, (e) quercetin, (f) ellagic acid, and a diluted solution of (g) tannic acid (2 µM). These antioxidants were chosen because they represent different classes of antioxidants of food interest (i.e., hydrophilic vitamins, lipophylic vitamins, polyphenols). Table 1 sums up their kinetic parameters, also in comparison with % of inhibition obtained with the classical DPPH• assay. All compounds were much slower antioxidants than ascorbic acid as their rate constants were from 10 to 500 times lower than that of ascorbic acid.

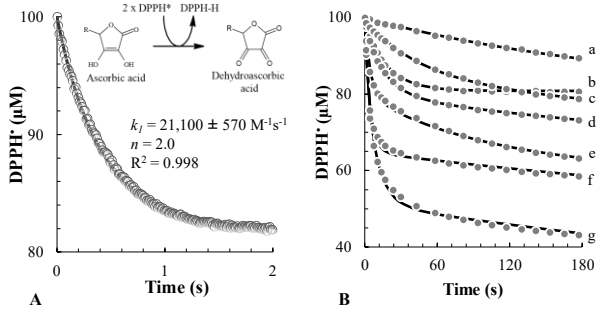

Figure 2 *A – DPPH• absorption decay in the reaction with 10 µM of vitamin C; B – kinetic curves for the reactions between DPPH and phloretin (a), vitamin E (b), catechin (c), epicatechin (d), quercetin (e), ellagic acid (f), and tannic acid (g).*

Ascorbic acid was the fastest (k_1 = 21,100 ± 540 $M^{-1}s^{-1}$), while phloretin the slowest (k_1 = 45 ± 9 $M^{-1}s^{-1}$), while tannic acid showed the highest capacity (n = 24), followed by ellagic acid (n = 3.7). The DPPH• kinetic model can rank antioxidant standards according to their activity and capacity.

7.4 Application of the kinetic model on South Tyrolean foods

The standards are necessary to determine the robustness of a method, but it needs to work on matrices to prove its efficiency. Thus, the DPPH• kinetic model was applied and tested on several South Tyrolean food extracts, such as aromatic herbs, fruit juices and, of course, apples. All the extracts were standardized for the initial concentration of phenolic compounds prior to analysis, so that results could be compared. Three different applications are reported in figure 3.

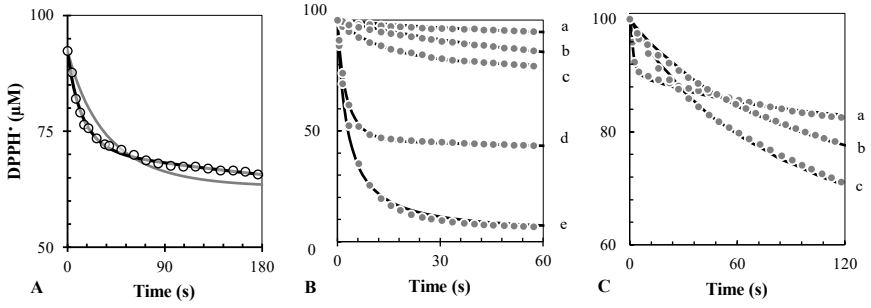

Figure 3 *A – kinetic curve for the reactions between DPPH• and 30 µM GAE of Harphagophytum Procumbens extract: fitting with the side reaction (dark line) vs fitting without the side reaction (light line); B – kinetic curves*

for the reactions between DPPH• and apple (a), red plum (b), peach (c), strawberry (d), and kiwi (e); C – kinetic curves for the reactions between DPPH• and Majda apple (a), Kissabel (b), and Golden (c).

The model successfully described the antioxidant behavior of all the food products tested. Overall, this approach could represent a novel tool to describe the quality of a natural extract. However, it gives no information about the composition in antioxidant compounds and which molecule is more responsible for the antioxidant behavior.

7.5 A more in-depth look: from food products to antioxidant compounds

The utility of the DPPH• kinetic model is that it can describe the antioxidant behavior, but other more sophisticated techniques, such as mass spectrometry and electrochemistry, should be employed to determine what are the compounds responsible of such characteristic. In the case of the three apples, the non-browning variety showed the lowest amount in phenolic compounds, but higher antioxidant activity than the red-flesh variety and Golden. Instead, the k_1 and k_2 values for Kissabel and Golden were not significantly different. For this reason, their composition was investigated. Apparently, the non-browning variety had a lower amount of phenolics, but its content in vitamin C was 4-5 times higher than in the other varieties. This finding explains the highest antioxidant activity of Majda with respect to the other varieties (Cebulj et al. 2023). Moreover, the anthocyanins responsible for the color in the red-flesh variety showed no reactivity in the electrochemical detector, explaining the similarities with Golden in the kinetic parameters.

8. Conclusions and Future Perspectives

The present study proposed a new fast, standardized and robust approach to assess the antioxidant activity and capacity of south Tyrolean food products. Moreover, the DPPH• kinetics combined with more complex tools, such as mass spectrometry and electrochemistry represents a comprehensive way to analyse the antioxidant behaviour of a food item. Thus, not only the overall activity and capacity of a matrix can be assessed, but also the determination of the molecules responsible for it. This research could help food industry to assess the quality of a product through the study of its antioxidant behaviour. Future studies should focus on the research of interaction mechanisms between the molecules.

9. Nomenclature

DPPH• = 2,2-diphenyl-1-pycrylhydrazyl; TPC = total phenolic content; AH = antioxidant; A = oxidized form of the antioxidant; DPPH-H = reduced form of the DPPH radical; GAE = gallic acid equivalents; LC = liquid chromatography; MS = mass spectrometry; CAD = coularray detector.

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Microbial exopolysaccharides as postbiotics for the development of new functional foods

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This PhD project aims to isolate, produce, and characterize new exopolysaccharides (EPS) of microbial origin. The EPS will be chemically characterized, and bioactivity and technological properties will be evaluated. Finally, EPS will be added to a food product and the effect on the gut microbiota will be evaluated.

Esopolisaccaridi di origine microbica per lo sviluppo di nuovi alimenti funzionali

Lo scopo di questo progetto di ricerca è quello di isolare, produrre e caratterizzare nuovi esopolisaccaridi (EPS) di origine microbica. I polisaccaridi verranno caratterizzati da un punto di vista strutturale, tecnologico e in termini di bioattività. Infine, gli EPS verranno utilizzati nella formulazione di un prodotto alimentare e verrà valutato il loro effetto sul microbiota.

Keywords: Exopolysaccharides, lactic acid bacteria, chemical characterization, technological properties, bioactivities.

1. Introduction

Following the PhD thesis project previously described (Bisson, 2021), this oral communication reports the main results of the following activities:

A1) Determining the chemical and morphological features of one EPS (EPS_O) produced by *Leuconostoc mesenteroides* strain Lm_O through NMR, FT-IR, and Scanning Electron Microscopy (SEM)
A2) Assessing the biological activities of EPS_O (antimicrobial, antibiofilm, and antioxidant)
A3) Assessing the technological properties of EPS_O (water holding capacity (WHC), oil holding capacity (OHC), solubility, rheology, …)

2. State of the art

In recent years, there has been increasing interest in microbial EPSs due to their technological properties and potential health benefits. EPS are large polymers produced during fermentation by many microorganisms, including lactic acid bacteria (LAB) (Yang et al., 2023). They can be classified into homopolysaccharides (HoPS), composed of a single type of sugar, or heteropolysaccharides (HePS), consisting of repeated units of different sugars (Yildiz and Karatas, 2018). *Leuconostoc* spp. is a LAB genus known for its ability to produce HoPSs, such as α-glucans and β-fructans (e.g., dextran and levan) (Bisson et al., 2023; Taylan et al., 2019). Dextran is a versatile EPS made up of α-(1,6) linked glucose units with varying degrees of branching. This polymer is commonly used in the medical and food industries due to its water retention capacity, solubility, and unique rheological properties (Díaz-Montes, 2021). Moreover, dextran has shown to possess also interesting bioactivities (prebiotic, anti-inflammatory) (Zarour et al., 2017). *Leuconostoc* strains produce unique dextran with specific characteristics (Mw, degree of branching, …) which determines both the technological and biological properties. For this reason, the discovery of new EPSs with peculiar structures and bioactivities represents an interesting research field.

3. Materials and Methods

3.1 EPS production

Leuc. mesenteroides strain Lm_O was isolated from Italian semi-hard cheese and identified by partial 16S rRNA gene amplification. The ability to produce EPS was qualitatively assessed on Mayeux Sandine ed Elliker (MSE) agar. EPS (named from here on EPS_O) was produced in MRS-S broth (glucose replaced by sucrose 20 g/L) inoculated at 1% (v/v) with an overnight culture and incubated at 25 °C for 48 h in aerobic conditions. EPS_O was then recovered following the method of Wang et al. (2019). After freeze-drying, EPS_O yield was determined by gravimetry.

3.2 Structural characterization

The UV-Vis spectrum of EPS_O was acquired by Varian Cary 50 spectrophotometer (Agilent Technologies, Santa Clara, CA). Functional groups were analysed using an Alpha-P(ATR)-FTIR spectroscope (Bruker Optics, Milan, Italy) (4 cm^{-1} 32 scans). EPS_O was hydrolysed and monosaccharide composition was obtained using a 1260 Infinity HPLC system (Agilent Technologies) equipped with a quaternary pump autosampler, refractive index detector, and Ultra Amino column (250 mm, 4.6 mm internal diameter, 5 µm) (Restek S.r.l., Cernusco sul Naviglio, Italy). The mobile phase was an acetonitrile/MilliQ water mixture (70:30, v/v), and the flow rate was 1.0 mL/min.

The NMR spectra were acquired using a Bruker Avance III 400 MHz digital NMR spectrometer (Bruker, Karlsruhe, Germany). NMR DOSY experiments were used to estimate the Mw of the EPS building a calibration curve using dextran standards (Mw 1.16×10^3 - 6.68×10^5 g/mol). The surface morphology was observed by an EVO 40 scanning electron microscope (Carl Zeiss, Jena, Germany) (SEM) equipped with energy dispersive x-ray spectroscopy (EDXS).

3.3 Antimicrobial and antibiofilm activity

Antimicrobial activity was tested against *Staphylococcus aureus* DSA 226, *Salmonella enterica* spp. *arizonae* DSMZ 9386, *Escherichia coli* DSA 8048, *Listeria monocytogenes* Scott A, *Listeria monocytogenes* APC 154, *Listeria monocytogenes* 1325, and *Enterococcus faecium* DSMZ 2146 following the method described by Bisson et al. (2023). Antibiofilm activity was measured following the protocol by Stepanović et al., (2000) against *Listeria monocytogenes* 284 and *Pseudomonas fluorescens* DSA L22.

3.4 Antioxidant activity

The antioxidant activity of EPS_O (0.5, 1, 2, 4 mg/mL) was assessed using the 2-2-diphenyl 1-pycrilhaydrazyl (DPPH) assay (Li et al., 2022) and the 2,2'-azino-bis (3-ethylbenzothialzoline-6-sulfonic acid) (ABTS) assay (Re et al., 1999). Ascorbic acid was used as positive control.

3.5 Technological properties

The Water Solubility Index (WSI) of EPS_O (5% w/w) was determined following the method of Saravanan et al. (2016). WHC and OHC were assessed following the method of Gan et al., (2020) resuspending 50 mg of EPS_O in 1 mL of water and oil, respectively. The thermal properties were evaluated using a TA4000 differential scanning calorimeter connected to GraphWare software TAT72.2/5 (Mettler-Toledo, Greifensee, Switzerland). The sample (2.5 ± 0.1 mg) was subjected to a heating ramp of 5 °C/min from 25 °C to 90 °C under a nitrogen flow maintaining the temperature for 1 min before cooling to 25 °C and finally heating from 25 °C to 120 °C. The glass transition temperature (Tg), the onset temperature (To), peak temperature (Tp), end-set temperature (Te), and transition enthalpy (ΔH) were determined using the software STARe ver.8.10 (Mettler-Toledo). EPS_O was dispersed in water at 5% (w/w) and its viscoelastic properties were evaluated using a Haake RheoStress 6000 controlled stress rheometer (Thermo Scientific) equipped with a Peltier system for temperature control and parallel plate geometry (35 mm diameter, 1.0 mm gap). The flow behavior was measured by recording shear stress values when shearing the samples at an increasing shear rate from 0.01 to 100 s^{-1}. The linear viscoelastic region was detected by stress sweep tests performed from 0.1 to 100 Pa at a constant frequency of 1 Hz and 20 °C. A frequency sweep test was carried out at 20 °C between 0.1 and 10 Hz using a stress value included in the linear viscoelastic region. Storage (G') and loss (G") moduli were obtained.

3.6 Statistical analysis

All trials were carried out at least in duplicate. Values are reported as the means ± SD. Analysis of variance was performed to evaluate the significance of differences among means ($p<0.05$) by using R v.3.0.2 for Windows (The R foundation for statistical computing, Wien, Austria).

4. Results and Discussion

4.1 Strain identification and EPS yield

Leuc. mesenteroides strain Lm_O formed mucous and slimy colonies on MSE agar plates. The yield of EPS_O in MRS-S broth was 3.84 g/L.

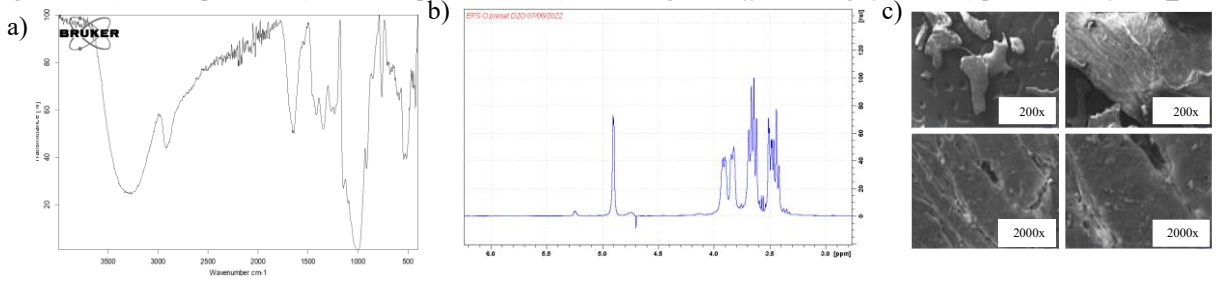
4.2 Chemical characterization

The UV-Vis spectrum didn't show the presence of peaks at 260 and 280 nm, indicating the absence of contaminants (proteins or nucleic acids). Monosaccharide composition analysis evidenced that EPS_O was constituted only of glucose units. FT-IR spectrum evidenced different peaks in the region between 3400 and 500 cm^{-1} (Fig 1a). The peak at 3000, 2000, 1600, 1640, 1149, and 1103 cm-1 corresponded to the stretching vibrations of O-H, C-H, water, C-O-C, and C-O groups respectively. The peak at 1000 cm^{-1} is due to the chain flexibility around the α (1→6) bond. The ^{1}H NMR spectrum, in the anomeric region, presented two signals at 4.9 and 5.3 ppm, corresponding to the branching D-glucoside residues α (1→3) and α (1→6) respectively (Fig 1b). The HSQC evidenced the α anomeric nature of the principal signals of EPS_O due to the presence of a cross peak at δH-1/δC-1 = 4.9/97.7 ppm. ^{13}C – DEPTQ and HSQC spectra allowed the identification of the main resonance at 65.5 ppm (δH-6,6′ = 3.69, 3.92) as the C-6 carbon, and the ^{13}C chemical shift demonstrated that the position is involved in the glycosidic linkage. The remaining signals in the range 69.5–73.4 ppm were assigned by homonuclear COSY and heteronuclear HSQC to the ^{1}H and ^{13}C bulk region, in agreement with previous data (Llamas-Arriba et al., 2019). The integration of ^{1}H anomeric signals highlighted that EPS_O has <5% of α (1→3) branching. The Mw of EPS_O was estimated with DOSY, and it was estimated > 6.68×10^5 g/mol. SEM images (Fig 1c) revealed that EPS_O has a compact structure. This tool is useful for understanding the physical properties of macromolecules (Insulkar et al., 2018). This result differs from others reported in the literature for dextran produced by other *Leuconostoc* species, indicating the heterogeneity of dextran-type EPS that this genus can synthesize (Zhao et al., 2019; Zhou et al., 2018).

4.3 Antimicrobial and antibiofilm activity

The effect of different concentrations of EPS_O on the growth kinetic parameters of specific microbial foodborne targets is reported in Table 1. EPS_O modified the growth kinetics parameters in different ways, depending on the pathogen tested.

Figure 1 *a) FT-IR spectrum, b) [1]H-NMR spectrum, and c) SEM images at different magnifications (up to 2000x) of EPS_O*



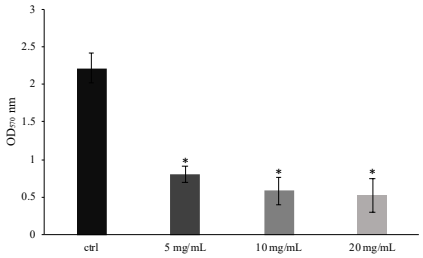
A statistically significant elongation of the lag phase was observed for *Salm. enterica* and *E. coli* in the presence of EPS_O, and the maximum growth rate was also reduced for *Salm. enterica*, *E. coli,* and *L. monocytogenes* at both the concentration tested. Indeed, the main growth parameter affected by the presence of EPS_O was the amplitude (i.e., the difference between the initial and the final OD). A statistically significant decrease in the amplitude was observed for almost all the pathogens tested (except for *E. coli*). The mechanisms underlying the antimicrobial effect of EPS are not clearly understood yet. It has been hypothesized that EPS could interfere with the cell membrane leading to its destruction, as well as cell division inhibition and DNA degradation could be responsible for the antimicrobial activity (He et al., 2010). Another speculation about the antimicrobial activity of dextran could be that due to its high Mw, the polymer could envelop the cells leading to an accumulation of secondary metabolites in the media, affecting the viability of the cells (Salachna et al., 2018). These results could open the door to the application of EPS as a new antimicrobial substance, whereas other studies are needed for understanding the mechanisms involved.

Table 1 *Effect of EPS_O on growth kinetic parameters of foodborne pathogens. For the same parameter and the same microorganism, asterisks indicate a significant difference ($p < 0.05$) with respect to the control*

microbial target	mg/mL	λ (h)	μ_{max} (log OD/h)	amplitude (log OD)
Ent. faecium DSMZ 20477	control	6.20±0.02	0.50±0.07	1.33±0.02
	2.5	6.49±0.70	0.45±0.03	1.15±0.10*
	5	7.10±0.80	0.64±0.02*	1.00±0.06*
Salm. enterica 9386	control	5.26±0.08	0.25±0.01	1.32±0.01
	2.5	5.21±0.12	0.16±0.08*	0.93±0.01*
	5	5.83±0.01*	0.19±0.03*	0.76±0.01*
E. coli 8048	control	5.65±0.04	0.41±0.02	1.33±0.04
	2.5	6.55±0.06*	0.23± 0.01*	1.18±0.02
	5	7.02±0.15*	0.30± 0.02*	1.24±0.02
S. aureus 226	control	5.90±0.18	0.29±0.01	1.42±0.03
	2.5	7.12±0.50	0.24±0.03	1.12±0.09*
	5	7.26±0.16	0.07±0.01	1.09±0.05*
L. monocytogenes Scott A	control	9.67±0.05	0.21±0.05	1.09±0.02
	2.5	9.49±0.23	0.12±0.06	0.75±0.07*
	5	10.25±0.13	0.10±0.05	0.48±0.03*
L. monocytogenes APC 1325	control	13.27±0.07	0.16±0.02	0.98±0.04
	2.5	13.50±0.04	0.12±0.01	0.56±0.01*
	5	13.39±0.05	0.11±0.01	0.51±0.06*
L. monocytogenes APC 154	control	13.44±0.15	0.23±0.05	1.21±0.01
	2.5	13.46±0.20	0.11±0.01*	0.56±0.03*
	5	13.53±0.12	0.11±0.04*	0.26±0.06*

EPS_O inhibited the biofilm formation by *L. monocytogenes* 284 (Fig 2), whereas no effect was observed against *P. fluorescens* L22 (data not shown). EPS_O slowed down the formation of *L. monocytogenes* biofilm also at the lowest concentration tested (5 mg/mL). These data hold significance since the presence of *L. monocytogenes* biofilm is a relevant issue in the food industry due to the possible cross-contamination. Indeed, it is well known that listeriosis exhibits a high fatality rate. On the other hand, EPS_O didn't inhibit the biofilm formation of *P. fluorescens*, probably due to the different nature of the biofilm (Marino et al., 2018).

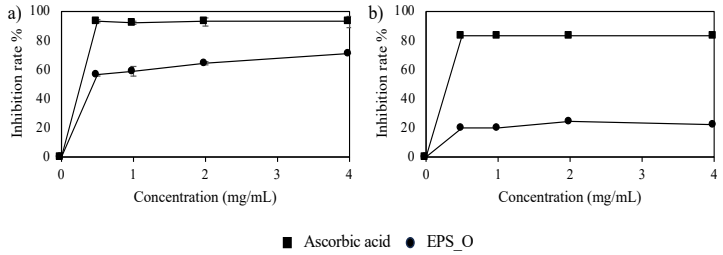
Figure 2 *Biofilm formation (OD_{570}) by L. monocytogenes 284 in the presence of EPS_O*



4.4 Antioxidant activity

The antioxidant activity was tested using two biochemical assays (DPPH and ABTS). In all cases ascorbic acid showed a stronger antioxidant activity. EPS_O demonstrated a good DPPH radical scavenging ability which increased as the concentration increased reaching a maximum of 71.33±3.73 % at the highest concentration tested (4 mg/mL) (Fig 3a). On the other hand, EPS_O demonstrated to exert a lower ABTS radical scavenging activity which reached the highest score (24.52±0.001 %) when EPS_O concentrations was 2 mg/mL (Fig 3b). These results could be of interest since other studies on the antioxidant activity of dextran reported lower radical scavenging activity (Bisson et al., 2023). These different results could be attributed to the different Mw of the polymers, since it has been previously reported that the Mw of dextran strongly influenced its antioxidant properties (Soeiro et al., 2016).

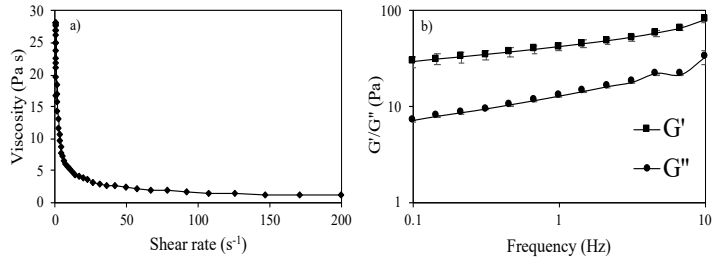
Figure 3 *Scavenging a) DPPH, b) ABTS activity of EPS_O*



4.5 Technological properties

The water solubility index (WSI), WHC, and OHC are important properties to be evaluated in considering the application of microbial EPS in food formulations. The solubility of EPS in water is strictly related to the length and degree of branching of the polymer and affects its dispersion and hydration. A high WHC and OHC are instead related to the ability of the molecule to hold water or oil influencing the technological performance of EPS and different food properties such as the retention of flavours and palatability. The WSI, WHC, and OHC of the EPS_O from *Leuc. mesenteroides* Lm_O were 99%, 784%, and 496%, respectively. Results confirmed the highly hydrophilic nature of the polysaccharide. Moreover, the polymer was extremely capable to retain water in its molecular structure, probably impacting its functional properties (emulsification, viscosity, gelation, …). The oil absorption capacity of EPS_O also makes it suitable for foods where the retention of flavour is desired (e.g., bakery products). The thermal stability of EPS_O was analyzed by DSC and results revealed that the melting peak was around 147 °C and the Tg at 83 °C, indicating the possible use of this polymer in foods where the use of high temperatures is required. The potential application of EPSs depends also on their rheological properties. In this study, EPS_O (5% w/w) showed a pseudoplastic behavior, as the viscosity decreased with the increase of the shear stress (Fig 4a). G' was always higher than G'' and frequency-dependent in the considered range indicating a weak gel behavior (Fig 4b). All these characteristics could highlight the potential use of EPS_O as a promising ingredient with specific technological properties for food applications.

Figure 4 *Viscosity-shear rate profile (a) and rheological properties G' and G'' (b) of EPS_O solution (5% w/w)*



5. Conclusions and Future Perspectives

Leuc. mesenteroides Lm_O produced a high Mw dextran that proved to exert exploitable bioactivities versus bacterial foodborne pathogens. EPS_O inhibited the growth and the biofilm formation of some of the microbial targets, suggesting the possible application of this polymer as a new antimicrobial agent. Moreover, the results on the antioxidant activity of EPS_O are encouraging, since a good DPPH radical scavenging activity was observed, suggesting its possible use as antioxidant in food products. EPSs are also known for their physicochemical attributes, improving the texture and palatability of foods. For this reason, is important to screen EPS with novel properties which can make these molecules of commercial value. The EPS analyzed in this study showed high WSI, WHC, and OHC, which make it a good candidate as texture improving agent in foods. Moreover, the dextran showed a non-Newtonian pseudoplastic behavior and it was able to form viscous solutions suggesting its possible use as a thickening and gelling agent.

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Soil spatial variability at vineyard scale and relationship between grape elemental profile and enological characteristics of Aglianico grapewine in Taurasi DOCG area

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This PhD thesis concerned the characterization of the SOIL-GRAPE-WINE system and the possible relationships between soil properties and enological characteristics/quality of grapes and wines. A multidisciplinary approach, including spatial, pedological and enological analyses, was applied to identify possible key factors of the soil-vineyard interaction to be used in the precision viticulture framework.

Variabilità spaziale del suolo a scala di vigneto e relazione tra profilo elementare dell'uva e caratteristiche enologiche del vitigno Aglianico nell'areale del Taurasi DOCG

Questa tesi di dottorato ha riguardato la caratterizzazione del sistema SUOLO-UVA-VINO e le possibili relazioni tra proprietà del suolo e caratteristiche enologiche/qualità delle uve e dei vini. È stato applicato un approccio multidisciplinare, comprendente analisi spaziali, pedologiche ed enologiche, per identificare possibili fattori chiave dell'interazione suolo-vigneto da utilizzare nel quadro della viticoltura di precisione.

Key words: Terroir; soil proximal sensors; grape elemental profile; grapes and wine enological characteristics.

1. Introduction

Identification of viticultural *Terroir* peculiarities enhances our understanding on why specific geographical areas contribute to the quality and uniqueness of wines. Indeed, the complex soil-plant interaction affects plant metabolism at different extent and, consequently, influences the enological characteristics of grapes and wines (White RE, 2020). The concept of *terroir* has been increasingly used to attribute a specific geographical connotation to products of excellence, such as wine. As stated by the OIV (International Organization of Vine and Wine) a viti-vinicultural *Terroir* is the result of the environmental influence of climate, geology and soil on vine behavior, in interaction with the variety, agrotechniques and wine making techniques (OIV/VITI Res. 333/2010). Since that, investigations on different environmental factors (i.e., climate, geomorphology, soil, rock) and their effect on grapes and wine quality have been performed by different authors (Van Leeuwen et al., 2018). However, less investigated has been the elemental profile of grapes in relationship with their enological properties, and the variability of both composition and enological characteristics because of the soil spatial variability at vineyard scale.

2. Environmental setting

This study focused on 3 vineyards (Vineyard A, B and C) from Irpinia land, all located in the clayey and marls hilly landscape of the Montemarano (AV) town, the core of Taurasi DOCG area, and applied on Aglianico red grape-variety. The aim was the identification of HZs at vineyard scale, in which sample and analyse grapes at two vintage times (2021 and 2022 years) for the identification of both their elemental profile (i.e., macro and micro element composition) and enological characteristics.

3. Materials and Methods

3.1 Vineyard selection

The 3 vineyards located at 483, 589, and 630 m a.s.l. respectively, presented the same VCR7 clone of grape variety Aglianico cv, with the same rootstock (1103P and 420A) used. The planting year, spurred cordon training system, and NE rows orientation are also consistent across the vineyards.

3.1 Proximal Soil Sensors (PSS)

Soil spatial variability assessment was performed by using PSS to identify and map HZs. In detail, Gamma Ray spectrometer was used to determine Th (ppm), U (ppm) and K (%) content, while electromagnetic induction (EMI) sensor was applied to determine apparent electrical conductivity (ECa) at different depths (50, 100 and 180 cm). Then, a portable X-ray fluorescence sensor was used to determine soil major and trace total element content. All

parameters were spatialised by ordinary kriging and mapped with QGIS software.

3.2 Pedological profiles: soil chemical and physical analysis

Pedological profiles and hand drillings were performed in the HZs, and sampling performed following soil horizons were sampled. Soil samples were analysed for their principal chemical and physical properties (including pH, Electrical Conductivity – EC, carbon content, total carbonates, cation exchange capacity - CEC, soil texture and bioavailable P_2O_5) following SISS methods (Analisi chimica del suolo (2021) FrancoAngeli Ed). In each HZs, grape sampling was addressed for both vintages 2021 and 2022.

3.3 Grape analysis

At harvest time, grape samples were collected to analyse enological parameters related to technological maturation. Parameters such as °Brix, pH, Titratable Acidity (TA), 50 berries weight and volume on 200 mL were determined immediately after harvest, on fresh samples. To assess the phenolic profile, phenolic compounds were extracted from skins and seeds using a simulated maceration process in a wine-like solution. The extracted liquid, enriched with phenolic compounds, underwent analysis using various techniques. High-Performance Liquid Chromatography (HPLC) was employed to quantify native anthocyanins, while Harbertson's assay was used to measure Total Anthocyanins, Bound Anthocyanins (LPP+SPP), BSA reactive Tannins, and Iron Reactive Polyphenols (IRPs). Spectrophotometric analyses determined the Color Index CI (sum of abs 420, 520, and 620 nm) and HUE (420/520 nm ratio). These analyses provided valuable insights into the phenolic composition of the grapes.

3.4 Nano-vinification experiments: wine analysis

Nano-vinification experiments were carried on the Vineyards A and B for the vintages 2021 and 2022. Grapes were sampled in two selected HZs for each vineyard, based on the maps obtained by the PSS. To standardize the fermentation conditions and minimize variations caused by natural yeasts present on the grape skins, the nano-vinification experiments involved the inoculation of the crushed grapes with a commercial yeast strain. Standardizing the fermentation conditions helps ensure reproducibility and allows for a more accurate evaluation of the impact of soil variations of the phenolic profile and other characteristics of the wines produced from grapes harvested in the different soil zones.

3.5 Grape elemental composition analyses

Grape skin, pulp and seeds were carefully separated, freeze-dried (skin and seeds) and homogenated after their digestion for 40 min in a microwave digester at 200°C with HNO_3. Grape elemental composition was then determined using ICP-OES Thermo-Fisher.

5. Results and Discussion

5.1 Mapping of Homogeneous Zones (HZs)

The use of PSS enabled to identify and mapping HZs within each vineyard. PSS data were interpolated by ordinary kriging and maps produced by QGis software. The maps reported in Figure 1 serve as a visual representation of the HZs within each vineyard, enabling a better understanding of the spatial variability of soil properties, which could potentially impact grape growth and development, as well as grape and wine quality and enological characteristics. Indeed, a more targeted sampling approach was implemented for the grapes in the vintages of 2021and 2022.

Based on the EMI maps, the following HZs were identified:

- Vineyard A: five different HZs
- Vineyard B: five different HZs
- Vineyard C: four different HZs

Maps of gamma-ray emissions are generally consistent with EMI, then the identified HZs were used to address

the pedological survey.

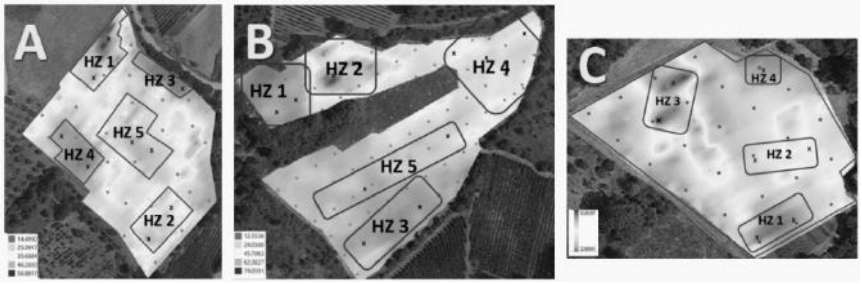

Figure 1 EC 180 cm maps which were utilized to identify and define the different HZs within the vineyards.

5.2 Soil chemical and physical properties

Results of soil chemical and physical analyses showed that Vineyard A, Vineyard B and Vineyard C were characterized by different soil properties.

Here is a breakdown of the findings:

Vineyard A:

The pH was moderately alkaline in Profile 1 (P1) (8.35 average value AV), Profile 2 (P2) (8.28 AV) and Profile 5 (P5) (8.16 AV), indicating a slight increase of alkalinity with depth. In Profile 3 (P3) and Profile 4 (P4) the pH showed values ranging between neutral and slightly alkaline (7.51 AV in P3 and 7.59 AV in P4). P3 and P4 exhibited the lowest pH among the mentioned profiles. Soil EC had an AV of 247.3 mS/cm in P1 (range from 127.9 to 483 mS/cm), an AV of 96.4 mS/cm in P2, (range from 102.3 on the surface to 90.8 mS/cm going deeper) and an AV of 127.1 mS/cm in P5 (range from 104.3 to 147.8 mS/cm). EC values in mentioned profiles showed a slight decrease with depth. Similar to the pH trend, EC values were higher in P1, P2 and P5. In P3 EC had an AV of 55.2 mS/cm (range from 33.2 to 76.4 mS/cm) and in P4 had an AV of 65.5 mS/cm (range from 48.4 in the deepest horizon to 87.4 mS/cm in the most superficial horizon). As for pH values, the lowest EC values found for P3 and P4. Regarding the C content, the AV was 4.24 g/kg and decreased with depth (ranging from 7.27 to 1.54 g/kg) in P1, 3.58 g/kg AV and range from 1.46 to 8.19 g/kg in P2, an AV of 3.07 g/kg with a range from 0.83 to 8.51 g/kg in P5. As expected, a regular decline of organic matter content was observed with depth for all the analyzed soil profiles, then no buried soils occur in the stratigraphic sequence. Moreover, P1, P2 and P5 showed the lowest values for C content. In P3, C content had an AV of 12.08 g/kg and ranged from 10.54 to 15.32 g/kg, while in P4, C content AV was 11.95 g/kg and ranged from 3.30 in the deepest horizon to 16.27 g/kg, which was the highest value among all the profiles in vineyard A. Then, the soil profiles showing the highest C content were in P3 and P4. TC content showed an average value of 68.69 g/kg in P1, an average value of 77.11 g/kg in P2, and an average value of 72.39 g/kg in P5. Interestingly, the trend of increasing values with depth was not consistently followed in P1 (ranging from 43.9 to 88.9 g/kg, with the lowest value recorded in the 120-155 cm horizon) and in P2 (minimum 29.7 g/kg at 60-70 cm, maximum 139.1 g/kg at 20-60 cm). This deviation from the expected trend suggested local variations in the carbonate distribution within the soil profile. In P5, the average TC was 72.39 g/kg with a range from 7.7 g/kg in the more superficial horizon to 174.4 g/kg in the deeper horizon. In P5, there was a clear tendency for total carbonates to increase as the depth of the horizons deepened.

In P3 and P4, there were the lowest values for TC, in accordance with pH, EC and C values. In P3, TC had an AV of 1.38 g/kg, which was the lowest among the mentioned profiles, while P4 had an AV of 2.30 g/kg.

Vineyard B:

Vineyard B exhibited a moderately alkaline pH (8.18 AV in P5, 8.30 AV in P1, 8.35 AV in P3, 8.39 AV in P2 and 8.42 AV in P4). EC values in P1 (76.9 mS/cm AV) and in P3 (89.8 mS/cm AV) were the lowest along the mentioned profiles, while in P5 (114.5 mS/cm AV), in P2 (149.5 mS/cm AV) and in P4 (205.2 mS/cm AV) values were higher. There were slight variations in the range of EC values, with a not constant trend to increase as the depth of the horizons deepen. C content showed the highest value in P1 (6.40 g/kg AV) and the lowest in P4 (2.55 g/kg AV), while in P2 (3.21 g/kg AV), in P3 (3.39 g/kg AV) and in P5 (3.29 g/kg AV) differences were slightly moderate. In P4, C content had the lowest value (2.55 g/kg AV) and showed a considerable range in the C content (from 0.56 to 9.50 g/kg). TC values didn't show consistent variations among the mentioned profiles (190.60 g/kg AV in P1, 150.60 g/kg AV in P2, 193.77 g/kg AV in P3, and 144.96 g/kg AV in P4). Only P5's TC value completely detached from the other profiles, with an AV of 10.64 g/kg. The lower pH, moderate electrical conductivity, lower carbon content, and lower total carbonates in P5 suggested a different soil composition compared to the other profiles of Vineyard B. Overall, Vineyard A showed more distinct differences between profiles in terms of pH, EC, TC, and C content. In Vineyard B, the differences were slightly more moderate, with variations in pH, EC,

TC, and C content across different profiles, but not as pronounced as in Vineyard A.

Vineyard C:

Soil pH in Vineyard C fell within the range of 8.09 to 8.83, indicating alkaline soil conditions. Particularly, HZ1 showed an average pH value of 8.35, HZ2 had 8.29, 8.42 in HZ3, and 8.45 in HZ4. The average EC values were 156.7, 180.7, 157.2, and 151.6 mS/cm, respectively, in the 4 identified HZs, with HZ2 having the highest EC value and HZ4 the lowest. The AVs of TC ranged from 127.1 to 117.3 g/kg in HZ1 and HZ2 and 90.3 and 366.5 g/kg in HZ3 and HZ4, respectively, the lowest and highest value of the entire vineyard C. Finally, the C content was 7.16 and 8.27 g/kg in HZ1 and HZ2 and 4.70 and 3.10 g/kg in HZ3 and HZ4. HZ3 had the lowest average TC value, while HZ4 had the highest value. C content was highest in HZ2 and lowest in HZ4. The pH values were relatively close, with HZ2 having the lowest pH and HZ4 having the highest.

5.3 Grape maturation indices

The results of the analysis of the main oenological parameters on technological maturation at harvest time for the vintages 2021 and 2022 in Vineyards A, B, and C are here reported.

Vineyard A:

- In both 2021 and 2022, the weight of 50 berries had the highest values in different HZs. In 2021, the highest values were observed in HZ3 (125.4 g) and HZ4 (119.0 g), while in 2022, the highest values were in HZ4 (130.9 g) and HZ5 (125.2 g).
- When comparing the two vintages, the 2022 vintage displayed slightly higher °Brix (24.3 in HZ1 and 24.6 in HZ2), higher pH (3.21 in HZ1, HZ3 and HZ5), and slightly lower TA (6.99 g/L) values compared to the 2021 vintage. It is also interesting to observe that there is a reverse trend in terms of °Brix in HZ1 and HZ2 comparing 2021 and 2022 values, 22.7 and 19.5 respectively in 2021, which are the lowest values in Vineyard A, 24.3 and 24.6 respectively in 2022, which are the highest values in Vineyard A.

Vineyard B:

- The differences recorded in various areas within Vineyard B remained in line between the two years (2021 and 2022).
- °Brix values were higher in HZ1 (25.1 in 2021 and 23.2 in 2022) and HZ2 (22.8 in 2021 and 23.5 in 2022), followed by HZ5 (23 in 2021 and 22.7 in 2022). The pH values were lower in HZ1 (2.99) and in HZ2 (2.97) in 2021 vintage and in HZ1 (2.98), HZ4 (3.00) and HZ2 (3.02) in 2022 vintage. TA values were higher in HZ1 in both vintages 2021 and 2022 (10.05 and 9.88 g/L respectively). Regarding the 50 berries weight, the lowest values were in HZ2 and HZ4 in both vintages (92.7 g in 2021 and 86.5 g in 2022 in HZ2, 99.4 g in 2021 and 100.6 g in 2022 in HZ4). HZ1 showed the highest values in 2021 (102.4 g), while in 2022 HZ1 (105.5 g) followed HZ5 (109.4 g).

Vineyard C:

- In Vineyard C, HZ3 and HZ4 exhibited the highest °Brix values (25.7 and 24.1 in 2021, 25.1 and 25.9 in 2022), indicating potentially higher sugar content in these areas.
- HZ1 and HZ2 in Vineyard C displayed the highest values for the weight of 50 berries (91.7 and 96 g in 2021, and 119.9 and 115.3 g in 2022), suggesting larger berries in these HZs. TA show the highest values in HZ2 and HZ3 (9.84 and 9.45 g/L) in 2021, and in HZ1 and HZ2 (11.27 and 9.41 g/L) in 2022.

These findings highlighted the variations in oenological parameters within different areas of each vineyard and the differences between the two vintages. Vineyard A showed differences in the weight of 50 berries between HZs and slight variations in °Brix, pH, and titratable acidity between the two years. Vineyard B exhibited quite high differences of parameters across HZs and years. Vineyard C displayed variations in °Brix and berry weight between HZs for both years.

5.4 Grape phenolic profile

The phenolic profile of the grapes in Vineyard A, B and C for the vintages of 2021 and 2022, was assessed to determine the extractability potential of the phenolic compounds. Additionally, certain parameters were measured to evaluate the phenolic content in different vineyard HZs. Here is a summary of the findings for each vineyard:

Vineyard A:

In 2021 vintage, HZ1 exhibited the highest values of CI (13.89), Tot Ant (1014.90 mg/kg ME), and most of native anthocyanins (delf-3mg 60.57 mg/kg ME, pet-3mg 79.91 mg/kg ME, malv-3mg 709.89 mg/kg ME). Additionally, BSA Tannins (553.13 mg/kg CE) and IRPs (1224.87 mg/kg CE) were also highest in this HZ. These results indicate that HZ1 had the highest concentration of phenolic compounds and the greatest extractability potential among all the HZs in Vineyard A. HZ2 had the lowest values of phenolic parameters compared to the other HZs in Vineyard A (CI 8.82, Tot Ant 595.96 mg/kg ME, delf-3mg 21.29 mg/kg ME, cian-3mg 2.33 mg/kg ME, pet-3mg 31.74 mg/kg ME, peon-3mg 22.89 mg/kg ME, malv-3mg 421.42 mg/kg ME, IRPs 691.19 mg/kg CE). HZ3 showed similarities to HZ4 in terms of phenolic profile. In detail, they did not have the highest values but shared some similarities in phenolic profile.

In vintage 2022, HZ1 and HZ2 tended to have higher values compared to the other HZs in Vineyard A (CI 12.63 and 13.84, Tot Ant 706.94 and 692.28 mg/kg ME, delf-3mg 47.60 and 42.43 mg/kg ME, cian-3mg 7.12 and 6.95

mg/kg ME, pet-3mg 60.23 and 55.65 mg/kg ME, peon-3mg 46.03 and 8.67 mg/kg ME, malv-3mg 457.13 and 448.75 mg/kg ME). In contrast to the previous vintage, HZ4 displayed the lowest values in terms of phenolic profile parameters (CI 9.40, Tot Ant 463.51 mg/kg ME, delf-3mg 21.33 mg/kg ME, cian-3mg 4.65 mg/kg ME, pet-3mg 25.58 mg/kg ME, malv-3mg 306.78 mg/kg ME, BSA Tannins 357.32 mg/kg CE and IRPs 741.83 mg/kg CE).
The findings suggest that there may be variations in the phenolic profiles of the grapes between vintages. Since HZ1 demonstrated higher values and extractability potential, the relative differences between the HZs differed in the two vintages.
Vineyard B: In both 2021 and 2022 vintages, HZ1 and HZ2 exhibit the highest values for various phenolic parameters, including CI (15.32 and 13.16 in 2021, 15.33 and 15.33 in 2022, respectively), Tot Ant (980.42 and 1010.88 mg/kg ME in 2021, 962.59 and 1029.95 mg/kg ME in 2022) and all native anthocyanins (delf-3mg: 86.19 and 72.84 mg/kg ME in 2021, 86.52 and 94.40 mg/kg ME in 2022, cian-3mg: 11.31 and 7.09 mg/kg ME in 2021, 11.19 and 9.97 mg/kg ME in 2022, pet-3mg: 101.12 and 91.58 mg/kg ME in 2021, 101.05 and 107.53 mg/kg ME in 2022). BSA Tannins and IRPs also demonstrate higher values in these HZs compared to the rest of Vineyard B. On the other hand, HZ3 consistently shows the lowest values for the entire anthocyanin component (CI 12.21 in 2021 and 11.49 in 2022, Tot Ant 730.10 g/kg ME and 686.97 g/kg ME in 2022 and native anthocyanins) in both 2021 and 2022 vintages. However, it's worth noting that Area 3 does not exhibit the lowest values for BSA Tannins IRPs, which suggests that the phenolic profile in terms of tannins and IRPs may differ from that of anthocyanins. The observed detachment of HZ1 and HZ2 from the rest of the areas in terms of higher phenolic content indicates their potential for greater extractability of phenolic compounds. Conversely, HZ3 consistently displays lower values for anthocyanins, suggesting lower concentrations and extractability potential for these compounds.
Vineyard C: In both 2021 and 2022 vintages, HZ3 and HZ4 consistently exhibit the highest values for CI (17.85 and 16.33 in 2021, 15.96 and 12.87 in 2022) and Tot Ant (1056.13 and 914.63 mg/kg ME in 2021, 795.78 and 667.98 mg/kg ME in 2022). On the other hand, HZ1 and HZ2 consistently show lower and more similar values for all components of the anthocyanin profile (CI, Tot Ant and native anthocyanins), as well as for BSA tannins and IRPs in both the 2021 and 2022 vintages. This suggests that these HZs have lower concentrations and extractability potential for phenolic compounds compared to HZ3 and HZ4.

5.6 Wine

The analyses of wines from Vineyard A and Vineyard B in the 2021 and 2022 vintages showed clear differences in enological parameters, as well as for the chromatic profile. These differences include alcohol content, pH, TA, and chromatic profile (CI, Tot Ant, tannins BSA, IRPs). However, comparing the vintages revealed that the variations in the chromatic profile were mainly due to the different stages of wine aging during analysis. Wine aging plays a crucial role in modifying color and phenolic compounds. As wines age, phenolic compounds undergo reactions affecting CI, HUE, and stability. Aging allows integration and polymerization of tannins, resulting in color stability and complexity. Phenolic compounds can interact with oxygen and other components, leading to pigment formation. Considering the wines' aging stage is important when comparing vintages and interpreting chromatic profile differences. These findings provided insights into wine evolution over time and helps understand how soil zones in Vineyards A and B contribute to phenolic composition and chromatic characteristics during different aging stages.

6. Conclusions and Future Perspectives

The obtained results indicate that among the identified HZs, those characterized by lower soil carbon content, higher EC, higher soil pH, and higher total carbonates revealed that were associated to favorable grape enological characteristics. These HZs displayed trends such as lower grape weight, higher °Brix, CI, Tot Ant, BSA tannins and IRPs values. These conditions suggest moderate vine stress contributing to the synthesis of desirable extractable phenolic compounds. In conclusion the differences recorded in HZs in terms of soil properties yield grapes with better enological attributes, including the chromatic profile and the phenolic composition, increasing the aging potential in wines.
Then, this approach revealed effective for the identification of vineyard areas characterized by different enological properties and elemental profile, giving a contribution in the assessment of precision viticulture methodologies aimed to wine quality improvement. Indeed, acquisition of knowledge on the enological variability at vineyard scale represent an additional possibility for winemakers to tailor their management strategies according to the specific soil characteristics.

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"Chimeric recombinant protein of *Brucella melitensis* and Immunological Evaluation for its possible use for the diagnosis of milk"

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Brucellosis is a disease caused by intracellular Gram-negative bacteria, which belongs to the *Brucella* genera and is distributed worldwide. In humans, the infection is easily transmitted through the consumption of unpasteurized milk or fresh cheese. The diagnosis is based on serological methods, which are not always sensitive or specific due to cross-reactivity with other bacterial antigens. We evaluated single variable domain on a heavy chain (VHH) antibodies against the chimeric recombinant protein BruD, which contain the epitopes Omp25, Bm26 and the Omp31, in milk artificially infected using the DotBlot assay. The antibodies VHH anti-BruD recognize the strain *B. abortus* 1119-3 up to 60 ng, so it is promising for a better diagnostic anti-brucella test.

Development strategy

The aim of the work was to test antibodies of Llama against a recombinant chimeric protein, for the development of serological strategies in the diagnosis of brucellosis with application in foodborne illness. The work was developed by our group in Venezuela during the first part of my PhD. We were focused in the bioinformatics analysis in the *omp31* gene, for the identification and selection of the epitopes from *Brucella melitensis* M15, *Brucella abortus* RB51 and *Brucella abortus* 1119 to design a chimeric protein BruD, subsequently the protein was expressed in a BL21 (DE3) system, using pET28a vector, and isolated by polyhistidine-NI affinity chromatography. The, polyclonal antibodies (VHH) against BruD were produced in Llama and evaluated by ELISA indirect and western blot against, *Brucella melitensis* M15 with high sensibility.

Key words: *Brucella abortus*, *Brucella melitensis*, brucellosis, milk, Indirect Methods

1. Introduction

Brucellosis is a zoonosis of food origin that is widespread worldwide, but rare in Europe, being caused by *Brucella* spp.. *Brucella melitensis* and *Brucella abortus* are gram-negative, facultative intracellular bacteria classified within the genus *Brucella* [1-3]. It is transmitted to humans through contact with infected livestock or by consumption of farm animal products. In humans, it causes weakness and debilitating febrile illness, known as an undulant fever by chronic infections, or aborts and infertility in bovines and other animals, resulting in severe economics losses and public health problems [4]. The more common methods for the detection are serological assays against bacteria's liposaccharides as ELISA test, as well as standard tube agglutination test (STAT), and the rose bengal plate agglutination test (RBPT). In Venezuela, the RBPT is used as a serologic detection method and STAT as a confirmation method test for brucellosis´s diagnosis [5]. The RBPT is non-expensive but low specific, therefore, the development of a faster and reliable method for brucellosis detection is quite important for foodborne illness.

2. Experimental Procedure

2.1 Evaluation of the use of IgG VHH for its application in the detection of *Brucella abortus* 1119-3, through a DotBlot.

To perform the Dot-blot assay, we used two different hardenings: BruD recombinant protein, from 90 ng diluted 1:2 to 3 ng, and whole *Brucella abortus* strain 1119-3 bacteria, from 270 ng diluted 1:2 to 17 ng. Nitrocellulose membrane (0.45 mm PIERCE) cut with the following measurements: 10 cm long x 7.5 cm wide. The sensors were placed for each dilution per well and allowed to air dry, then the membrane was placed under constant agitation at room temperature for 3 hours in 0.2 M Na_2HPO_4, 0.5 M NaH_2PO_4, pH 7.5 blocking solution; 0.05 % v/v Tween 20 and 5 % w/v skimmed milk. The membrane was incubated at room temperature for 30 minutes under excitement in incubation solution with a 1:100 dilution with the main anti-BruD. It was then washed five times for 5 min using PBS-T PBS wash solution, 0.1% v/v Tween 20. The reaction with the secondary was initiated by diluting peroxidase-conjugated anti-llama IgG diluted 1:5000 in blocking solution in the same conditions, then the membrane was washed following the previous indications. Detection was performed with 10

mg diaminobenzidine in 20 ml PBS, 50 µl 1 % $CaCl_2$ and 7 µl 3 % H_2O_2 with gentle compression. The reaction was quenched with H_2O_d.

2.2 Evaluation of the use of a polyclonal anti-BruD Llama serum for its application in the detection of anti-*Brucella* sp. using a sandwich DotBlot.

The adequate amount of antibody to sensitize the nitrocellulose membrane was standardized, working with 10 µl of a 1:100 dilution of each positive and negative serum previously evaluated by ELISA. A concentration of 60 ng of *Brucella abortus* strain 1119-3 and BruD was used, a 1:1000 dilution of the anti-BruD VHH antibody and 1:5000 for the anti-llama conjugate. It was challenged with diaminobenzidine and in cases where the formation of a dark coloration was observed, it was considered a positive result (Fig 2). Clear recognition was observed with the volume of antibodies tested. In the case of the negative sample, it was established that a weak signal, such as the one shown in Figure 2, is the maximum that can be observed in a sample of this type, maintaining the membrane for 5 min in the presence of the solution.

2.3- Sandwich DotBlot against *Brucella* 1119-3 in skim milk

A variant of the Dot-Blot similar to the previous described was carried out, where in order to assess the usefulness of the Llama anti-BruD polyclonal antibody to capture Brucella abortus strain 1119-3 bacteria in skimmed milk artificially contaminated with the bacteria. In this assay, we used positive and negative bovine antibodies to be fixed on a nitrocellulose membrane, after blocking, we placed 20 ng of Brucella abortus 1119-3 bacteria in skimmed milk diluted to 5 %, incubated for 1 h, washed with PBS and then incubated with 100 µl of the antibody purified by BruD immunosorbent. To determine its presence, it was incubated with the anti-flame conjugate.

3. Materials and Methods

In order to evaluate the potential of Llama single chain antibodies as a tool for the detection of *Brucella* sp. the DotBlot assay was performed. The evaluation process of the antibodies against the bacterial suspension *B. abortus* strain 1119 -3 and BruD was carried out in order to verify if the method works against the antigen with which the antibodies were induced. Higher to lower concentrations of strain 1119 and recombinant BruD diluted in 5 % skim milk and PBS-Tween solution were stained on a nitrocellulose membrane. Then, the VHH anti-BruD antibody was used at a 1:1000/well dilution. To detect this antibody, 1/5000 peroxidase-conjugated anti-Llama conjugate was placed in PBS-Tween. Then developed with diaminobenzidine.

4. Results and Discussion

4.1 - Evaluation of the use of IgG VHH for its application in the detection of Brucella abortus 1119-3, through a DotBlot.

As we can see in the **Figure 1**, using polyclonal tools as detection manifestations in the DotBlot assay, it was able to capture or detect the antibodies present on the surface of the nitrocellulose membrane in presence of skim milk.

Figure 1 Dot-Blot assay. Detection test for *Brucella abortus1119*-3 and BruD antigen using an anti-BruD Llama polyclonal Nitrocellulose paper was sensitized from higher to lower concentrations of strain 1119-3 and BruD. Anti-Llama peroxidase conjugated antibody was used at a 1/5000 dilution. In A, recognition of Strain 1119-3 is observed up

to a concentration of 17 ng and in B of BruD up to 5.5 ng.

This result means that Llama's tests confirm the epitopes of the Omp25, Bm26 proteins and the Omp31-like protein present in the bacterium *Brucella abortus* strain 1119-3.

4.2 Evaluation of the use of a polyclonal anti-BruD Llama serum for its application in the detection of anti-Brucella sp. using a sandwich DotBlot.

Clear recognition was observed with the volume of antibodies tested. In the case of the negative sample, it was established that a "faint" signal, such as the one shown in figure 2, is the maximum that can be observed in a sample of this type, maintaining the membrane for 5 min in the presence of the developing solution.

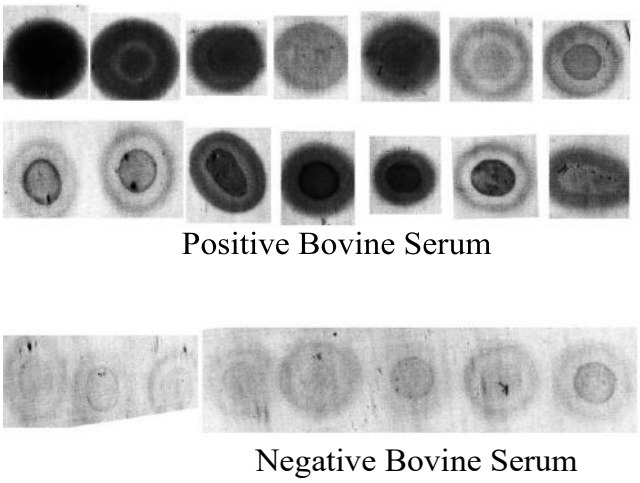

Figure 2 Sandwich Dot-Blot assay. Control sera previously evaluated by the ELISA test were evaluated. Positive serum give an evidently positive signal when compared to negative serum**.**

4.3 Sandwich DotBlot against Brucella 1119-3 in skim milk.

The Dot-ELISA technique showed to be sensitive and accessible, since it does not require specialized equipment as in the case of ELISA, Western-blot or indirect immunofluorescence, among other techniques. The interpretation of results is simple and the recombinant BruD protein can be used as antigen, the Dot-ELISA technique is a diagnostic technique that could be used as a first screening in endemic areas or for field studies. A graphic description of the technique is shown in Figure 3.

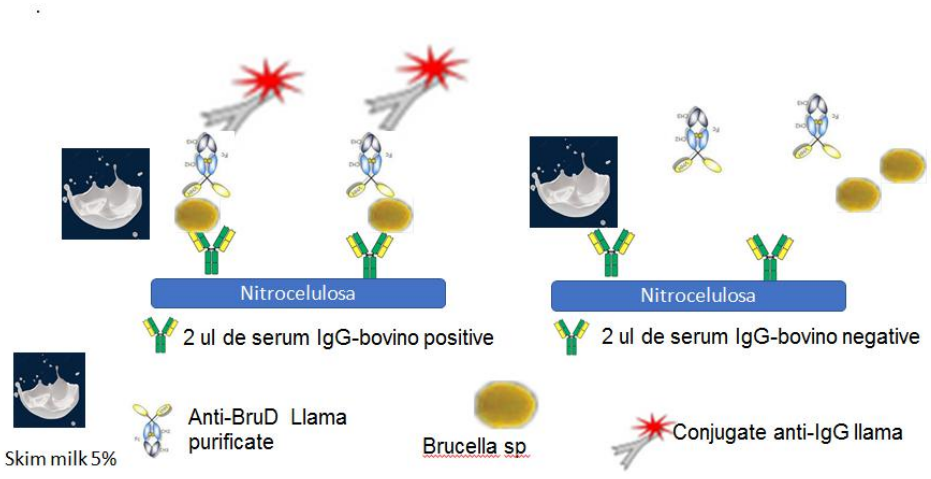

Figure 3 **Graphic of a Dot Blot** type immunological test using anti-Brucella sp. of bovine fixed to nitrocellulose and as antigen the strain of Brucella 1119-3 in milk artificially contaminated milk.

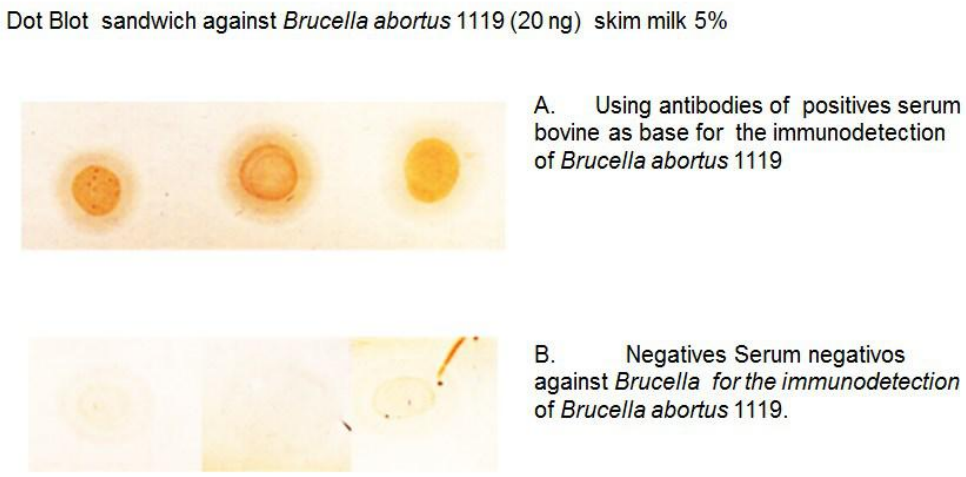

Figure 4 **Dot Blot sandwich against *Brucella abortus 119* (20ng) in skim milk.** shows that positive sera clearly capture the bacteria in the presence of artificially contaminated skimmed milk, while negatives do not. It is a qualitative test that demonstrates the usefulness of "VHH" single chain antibodies for the diagnosis of *Brucella sp.*

6. Conclusions and Future Perspectives

The use of policlonal antibodies of Llama antiBruD could be improved in the level of specificity for being used in the development of serological assays as DotBlot. However, the use of VHH policlonal simple chain is a novelty. This achievement will undoubtedly allow commercial independence in relation to antigen production, taking a big step at the institutional level on the path towards the production of diagnostic kits against *Brucella* in foodborne illness.

8. References

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Biotechnological Valorisation of Residues and By-Products From Agro-Food Industries

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This PhD thesis dealt with the valorisation of some agro-food by-products (BPs) (i.e. pomegranate and citrus peel and seeds, and field horsetail) to use them as food ingredients. The BPs were initially characterised for their chemical (i.e. phenolic and volatile profiles) and functional (i.e. antimicrobial, prebiotic and antioxidant) properties. Citrus and horsetail fortified "Primo Sale" cheeses and a beverage added with functional lactic acid bacteria (LAB) and pomegranate BPs were then formulated. Finally, residues from clementine juices were valorised through microbial fermentation using selected yeasts and LAB to obtain an ingredient for a plant-based beverage.

Valorizzazione biotecnologica di residui e sottoprodotti dell'industria agro-alimentare

Questa tesi di dottorato ha riguardato la valorizzazione di alcuni sottoprodotti agroalimentari (bucce e semi di melograno e agrumi ed *Equisetum*) per utilizzarli come ingredienti. I sottoprodotti sono stati caratterizzati per le loro proprietà chimiche (profilo fenolico e molecole volatili) e funzionali (attività antimicrobica, prebiotica ed antiossidante). Sono stati poi formulati dei formaggi "Primo Sale" fortificati con residui di agrumi ed equiseto, e una bevanda addizionata di melograno e batteri lattici. Infine, i residui della produzione di succhi di clementina sono stati valorizzati mediante fermentazione microbica con microrganismi selezionati per ottenere un ingrediente per una bevanda a base vegetale.

Key words: By-products; pomegranate and citrus peels; *Equisetum arvense*; "Primo Sale" cheese; microbial fermentation.

1. Introduction

In accordance with the PhD thesis project previously described, this oral communication reports the main results of the following activities directed to:

A1) Chemical and functional characterization of the by-products (BPs);
A2) Use of citrus BPs and horsetail in "Primo sale" cheese production;
A3) Formulation of a probiotic beverage with added pomegranate BPs;
A4) Valorisation of clementine BPs through microbial fermentation.

2. By-products in the food industries

According to Eurostat (Agriculture, forestry and fisheries statistics, 2020), up to 14% of food produced globally undergoes quantitative food loss. Food Loss and Waste (FLW) affects the sustainability of food systems, with negative impacts on the economy, food security, nutrition, and the environment. FLW is an important global issue and is linked to the Sustainable Development Goals (SDG) 12 - Responsible consumption and production - and 2 – Zero Hunger. Nowadays, the major uses of wastes and by-products (BPs) include animal feed or energy production, as well as the extraction of some high value-added products. However, most BPs are unused and discarded (Maurya *et al.,* 2015) despite being rich in substances which can be recovered, e.g. simple and complex sugars, lipids, proteins, micronutrients, essential oils, and dietary fibre (Mejri *et al*., 2018). Over the past decades, attention to diet and health has strongly increased, and the consumption of low-fat, low-calories, fibre- and antioxidant-rich foods has attracted the attention of many consumers as they significantly contribute to the reduction of cholesterol and the prevention of cardiovascular diseases and constipation (Aslam *et al.*, 2014). The use of food processing BPs in traditional and innovative foods can improve their functional properties, e.g. by increasing their fibre content, antioxidant or prebiotic activity, as well as extending their shelf-life. In fact, the food industry is constantly looking for new strategies, including the replacement of traditional preservatives with natural compounds, to satisfy the consumer's desire for fresh and 'natural' products, while maintaining food safety, quality and stability standards.

For these reasons, two different BPs were chosen, i.e. those from the citrus fruit industry (orange and clementine peels and seeds) and those from pomegranate (peels and seeds). Citrus fruits are widely cultivated with 14.49 million tonnes produced worldwide in 2019 (FAOSTAT, 2020). Unlike other fruits, the edible portion of citrus fruits is low compared to the inedible one: in fact, the latter is about 50-60% and comprises mainly seed, peel

and juice extraction residues. The world production of pomegranate turns out to be about 6 million tonnes per year of which about 40% is peel. Thanks to the recovery of antioxidant-rich extracts, many commercial applications are available for pomegranate peel derivatives, including their use in pharmaceuticals, in tinctures and foods, in anti-cancer therapeutic agents and the synthesis of copper oxide (Witt *et al.*, 2022). In addition, a weed, *Equisetum arvense* or field horsetail, was also included in the PhD project as it is used for phyto-therapeutic purposes for internal use to treat inflammation of the oral cavity, tonsillitis, acne, cold sores and other afflictions due to its reported bioactivities, e.g. astringent, diuretic, anti-inflammatory, antibacterial, antimicrobial and antioxidant properties.

3. Experimental Procedure

This PhD thesis was organised into 3 different steps: i) chemical and functional characterization of the different BPs; ii) use of the BPs as ingredients directly added to "Primo Sale" cheeses or beverages; iii) set up of a biotechnological process to enhance functional characteristics of clementine BPs. The first step was essential to determine the polyphenols and volatile profiles of the different BPs, and to assess their antimicrobial and prebiotic activity. After this step, the most promising BPs were used as ingredients directly added during the production of different foods. In particular the horsetail and the orange peel were added to 'Primo Sale' cheeses, while the pomegranate residues were used for the production of beverages added with probiotics. Finally, the clementine BPs were fermented by different strains of bacteria and yeasts to enhance some functional and technological features, and to obtain an ingredient which can be used in a plant-based beverage.

4. Materials and Methods

The BPs samples used during my PhD thesis are listed in Table 1.

Table 1 *BPs used during my PhD thesis.*

Common name	Scientific name	Family	Origin	Year	Examined product	Sample Code
Orange	*Citrus sinensis*	*Rutaceae*	Emilia Romagna	2012	Peel-pomace	Cit12
Orange	*Citrus sinensis*	*Rutaceae*	Emilia Romagna	2021	Peel-pomace	Cit21
Clementine	*Citrus clementina*	*Rutaceae*	Reggio Calabria	2023	Peel-pomace	Clem
Field horsetail	*Equisetum arvense*	*Equisitaceae*	Emilia Romagna	2019	Leaves	Eq19
Field horsetail	*Equisetum arvense*	*Equisitaceae*	Emilia Romagna	2021	Leaves	Eq21
Edible Pomegranate	*Punica granatum*	*Lythraceae*	Emilia Romagna	2022	Peel	PEE
Edible Pomegranate	*Punica granatum*	*Lythraceae*	Marche	2022	Peel	PEM
Edible Pomegranate	*Punica granatum*	*Lythraceae*	Marche	2022	Pomace	AM
Ornamental Pomegranate	*Punica granatum*	*Lythraceae*	Marche	2022	Peel-seeds	POM

4.1 BPs characterization

Samples were air dried (Eq) or freeze-dried (Citrus and pomegranate BPs) and grinded. According to Ferioli and D'Antuono (2016), the methanolic extracts were tested for total phenolic content (TPC, Folin-Ciocalteau assay), $ABTS^{\bullet+}$ assay and DPPH (0.1mM) scavenging activity (Abid *et al.,* 2017). The results were expressed as gallic acid equivalent (GAE (mg kg^{-1})), Trolox equivalent (TEAC) and IC50, respectively. The extracts were tested against 49 strains (starters/probiotics, pathogens and yeasts) belonging to the collection of DISTAL- University of Bologna, with the agar diffusion method (Rao *et al.*, 2016). The most sensitive ones were used to determine the Minimum Inhibitory Concentration (MIC) by microdilution broth method. The dried samples were also analysed for the volatile compound according to Tabanelli *et al.* (2013). Finally, the BPs were characterized for their prebiotic activity on two commercial probiotics, i.e. *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Lactiplantibacillus plantarum* DSM 25710, by determining the prebiotic activity score (Huebner *et al.,* 2007) and the ability of the BPs to protect and promote the probiotic growth in a simulated intestinal fluid (SIF; 0.15% w/v Oxgall bile salts in 100 mM phosphate-buffered saline pH 8 and subsequently sterilised and added with 0.1% w/v pancreatin; each BP concentration 50 mg cm^{-3}).

4.2 Citrus and *Eq* as ingredients for 'Primo Sale' cheeses

'Primo Sale' cheeses were prepared at lab scale with pasteurised whole milk and yoghurt as starters (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*). Three LAB strains (*Lactiplantibacillus plantarum* B39.1.4A, *Pediococcus pentosaceus* B39.2.2A, *Enterococcus faecalis* B39.2.2B) were also added to some samples (~7 log CFU g^{-1}). After coagulation and before breaking the curd, citrus (2% w/w) and Eq (1% w/w) were added and then the cheeses were formed. The samples were monitored for the viability of the native microbiota and of the starters, the changes in the aroma and TPC, the antioxidant activity and the color over one month of storage at 4°C.

4.3 Beverages added with pomegranate peels

24 different formulations of beverages were produced by mixing (1:1) apple juice and pulp and: i) whole milk (V); ii) soy beverage (S); iii) spelt beverage (F); iv) oats beverage (A). Half of the samples were also added with the pomegranate peels powder (0.7%, w/w), while the others were used as controls. All the samples (with or without pomegranate) were inoculated with two commercial probiotics separately (*Bifidobacterium animalis* subsp. *lactis* BB-12 and *Lactiplantibacillus plantarum* DSM 25710; initial inoculum rate ~8 log CFU cm^{-3}). The samples were stored at 4°C and monitored for the native microbiota and the viability of the probiotics. The samples were also analysed for TPC, antioxidant activity, prebiotic activity and for the ability to survive a simulated gastrointestinal digestion process.

4.4 Clementine peels valorisation by microbial fermentation

A mixture of grinded clementine peels and water (40:60, w/w) were inoculated with 12 LAB and 27 yeast strains (inoculum rates ~ 7 and 5 log CFU g^{-1}, respectively) belonging to the DISTAL Microbial Culture Collection. The growth ability of the strains, prebiotic activity, TPC, antioxidant activity ($ABTS^{\bullet+}$ and DPPH assay), and volatile compounds (SPME/GC-MS) of the fermented BP were evaluated.

5. Results and Discussion

5.1 BPs characterization

As previously described (Cellini, 2022), pomegranate peel extracts were the samples with the highest TPC values, while pomegranate seeds showed the lowest content with 112 GAE (mg kg^{-1}). Intermediate values were found for both Eq samples, close to 430 GAE (mg kg^{-1}) and citrus, 240 GAE (mg kg^{-1}). The TPC data were in agreement with the scavenging activity results. PEE, PEM and POM were found to be the most active BPs against all the microbial strains tested, especially against the pathogens. However, most of the tested yeasts seemed to be sensitive to Eq and Citrus, whereas LAB were not affected by the BPs. This result can be explained by considering the TPC values and the analysis of the volatile compounds of these BPs, which showed high amounts of terpenes, e.g. D-limonene in the Cit-samples and (S)-D-carvone in the pomegranate ones, which are widely recognised as antimicrobial substances (Aggarwal *et al.*, 2002).

When tested for their prebiotic activity on *L. plantarum* DSM 25710 and *B. animalis* subsp. *lactis* BB-12, the BPs were promising in that they were able to protect and promote their growth in SIF. In particular, all the tested substances increased the viability of both the strains by approximately 3 logarithmic cycles compared with the controls. It is important to note that POM, which was however significantly different from the control ($p<0.05$), gave rise to a reduction in cell viability of *L. plantarum* after 24 hours (Figure 1).

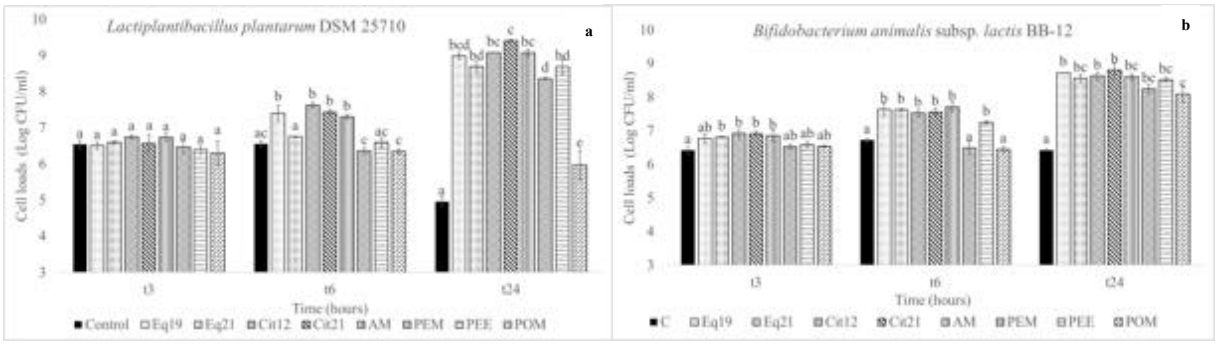

Figure 1 *Prebiotic activity in SIF of the different BPs on* L. plantarum *DSM 25710* (a) *and* B. animalis subsp. lactis *BB-12* (b) *after 3, 6 and 24 hours of incubation. Different letters mean significant differences between the time ($p<0.05$).*

5.2 'Primo Sale' cheese

The addition of the powdered by-products did not affect the viability of the starters, which remained above 8 log CFU g^{-1} over storage. On the other hand, an inhibition in the growth of some microbial groups such as *Enterobacteriaceae* and *Pseudomonas* spp. was observed (Figure 2). In particular, the presence of Eq and citrus resulted in a lower growth for *Pseudomonas* spp. by more than 3 logarithmic cycles compared to the control with the LAB, and more than 6 Log units compared to the control without them. As a consequence, *Pseudomonas* spp. never attained cell loads of 7 log CFU g^{-1}, which is considered a threshold for microbial spoilage, in cheeses added with the BPs.

The addition of the citrus BP resulted increased the TPC of cheeses of approximately 50 GAE (mg kg^{-1}). compared to the control samples. Furthermore, the phenolic content of all matrices increased over time, doubling at the end of storage (from ~150 to 330 GAE (mg kg^{-1})). This behaviour was also observed for the antioxidant

activity being significantly different ($p<0.05$) between samples at different storage times. The analysis of volatile molecules showed differences for several compounds, in particular in the content of the organic acids which increased during storage in the samples with the presence of the BPs. The same result was observed for short chain fatty acids (SCFA) such as acetic, butanoic and hexanoic acids which are well known to have benefits to the host when produced by the gut microbiota. Also, some authors reported that intake of dietary SCFAs protected against high-fat diet-induced obesity in mouse model (Caetano-Silva *et al.,* 2023). It is also relevant to point out that the citrus sample had a terpene content significantly higher (100 times) than the other samples ($p<0.05$), mainly due to the presence of D-limonene. Moreover, the samples with citrus had a volatile profile much richer compared with the control, which positively affected the overall aroma of the cheese.

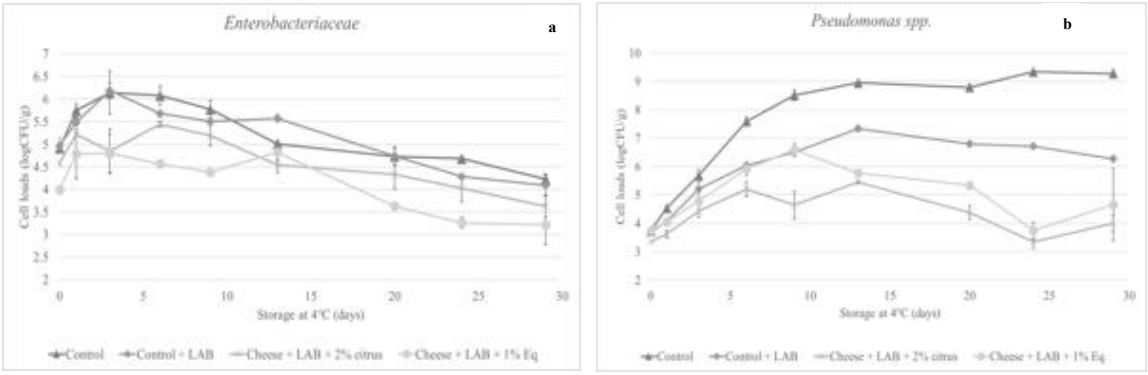

Figure 2 *Cell viability of* Enterobacteriaceae *(a) and* Pseudomonas *spp. (b) in 'Primo Sale' cheeses during a one-month storage at 4°C.*

5.3 Beverages added with pomegranate peels

No differences were detected in the viability of both *B. lactis* BB-12 and *L. plantarum* DSM 25710 5 hours after their inoculum in almost all the formulations regardless the presence of the pomegranate peel. During storage, cell loads of the probiotics remained stable (~ 8 log CFU cm^{-3}) or increased, although some differences were detected between the microbial species also in relation to the formulation. In particular, growth of *B. lactis* BB-12 was delayed in the oat beverage added with the pomegranate. However, following an initial adaptation it reached approximately the same level as the control sample (Figure 3a). On the other hand, *L. plantarum* was not affected by the presence of pomegranate (Figure 3b), not only in the formulation with whole milk, but also in the soy- and oat-based beverages. The prolonged lag-phase observed for *B. lactis* BB-12 may be due to a higher sensitivity to the terpenes and other components of the pomegranate BP having antimicrobial activity. In fact, according to data of the preliminary characterization, pomegranate peels were the BPs with the highest antimicrobial activity, also against the tested LAB.

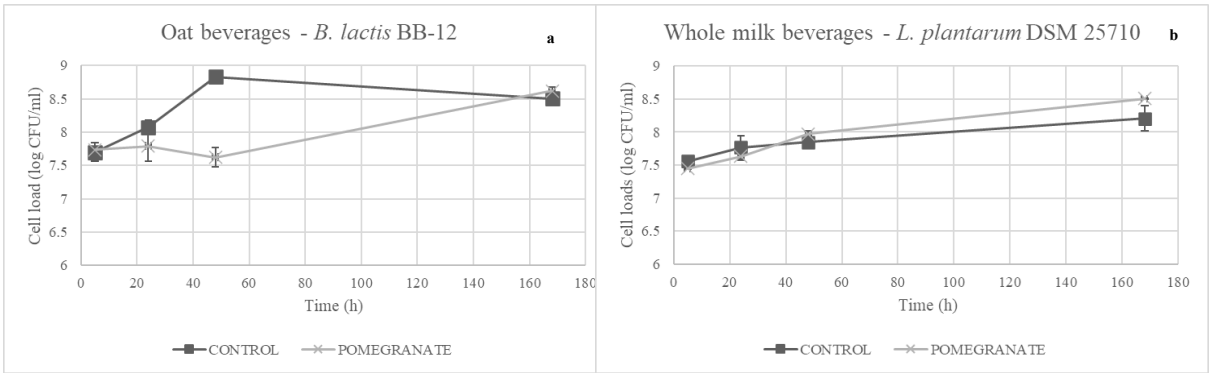

Figure 3 *Cell viability of the probiotics in two different formulations:* B. lactis *BB-12 in oat beverage and* L. plantarum *DSM 25710 in whole milk with (X) and without (■) pomegranate during a 7 days storage at 4°C.*

5.3 Clementine peels valorisation by microbial fermentation

A strain-dependent behaviour was observed over 7 days of fermentation. In fact, all the LAB strains and most of the yeasts (14 out of 19) were able to survive and grow with final increases of more than 2-3 log CFU g^{-1} on clementine residues without the addition of any other nutrient also considering that such a matrix presents rather stressing characteristics for microbial growth, e.g. low pH (3.5-4.0), organic acids, essential oils, poorly

fermentable carbohydrates. In general, microbial growth resulted in increases in the total phenol content, which are widely recognised to have anti-inflammatory and antimicrobial activity (Mo *et al.*, 2022). Moreover, the fermentation with the selected strains contributed to change the aroma of the clementine BP-based matrix. In general, in the first phase of the fermentation an accumulation of several alcohols and terpenes (e.g. linalool or alpha pinene) was detected. These compounds are responsible for flavours which are generally appreciated such as floral, citrus, sweety and minty. On the other hand, after 5 days of fermentation different profiles were observed among the strains with a general decrease in the content of most of the compounds detected in the early phase; on the contrary, molecules such as α-terpineol, terpinene-4-ol and ethyl acetate were accumulated. According to literature these terpenes show antimicrobial activity in addition to having positive aromas which can be exploited by using the fermented BPs as flavouring agents.

6. Conclusions and Future Perspectives

In conclusion, these experiments highlighted some potentialities of agro-food by-products and possible strategies for their valorisation as functional food ingredients. In particular, due to their composition rich in phenols, the tested BPs can be exploited as antimicrobials being active against different microbial species both *in vitro* and in the real system "Primo Sale". For the latter, a strong effect against specific microbial populations, i.e., *Enterobacteriaceae* and *Pseudomonas* spp. was detected thus contributing to the extension of the shelf life of the cheeses. In addition, the presence of the functional LAB, which remained viable over storage, can provide healthy features being the probiotic activity of cheeses enhanced by the presence of the BPs.
Moreover, the possibility to include the pomegranate BP into beverages promoted the viability of the two probiotic strains, thus increasing the overall functionality of the product also due additional features such as antioxidant activity, prebiotic effect due to the increased fibre content.
Finally, the possibility to valorise the clementine residues by microbial fermentation can be a solution to the stability of this type of BP and, at the same time, improve the availability of bioactive substances or provide new features as flavour and aroma, which can be important for the formulation of an innovative product.
This work considers only a few BPs and only on a laboratory scale; it will be interesting to evaluate the process on a pilot scale to better understand if they can be a real resource for the food industry. It will also be important to evaluate through panel and consumer tests the acceptance by consumers of the products added with the functional BPs.

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Formulation design strategies to increase the stability, quality and nutritional properties of frozen desserts

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Frozen desserts are complex systems whose quality and stability are highly dependent on their formulation and storage conditions. Product design approaches that consider the effects of the ingredients and related biomolecules on the final product's physical, physicochemical, colloidal and nutritional attributes need to be applied to maintain consumer acceptability.
This PhD project aims to design and develop formulations of frozen dessert-type products with improved quality, stability and nutritional value. The use of ingredients and biomolecules to stabilize the aqueous phase in the frozen state and the development of dairy- and egg-free product formulations are studied. The project is developed taking as a reference the mascarpone-based cream used as the main ingredient in the Italian typical dessert tiramisu.

Strategie di formulazione per migliorare stabilità, qualità e proprietà nutrizionali di semifreddi congelati

I dessert congelati sono sistemi complessi la cui qualità e stabilità dipende dalla formulazione e dalle condizioni di stoccaggio. Per mantenere l'accettabilità del consumatore, è necessario applicare approcci di progettazione della formulazione, che considerino l'effetto di ingredienti e relative biomolecole sugli attributi fisici, fisico-chimici, colloidali e nutrizionali. Questo progetto di dottorato ha l'obiettivodi progettare e sviluppare formulazioni di dessert con maggiore qualità, stabilità e valore nutrizionale. Vengono studiati l'uso di ingredienti e biomolecole per stabilizzare la fase acquosa in stato congelato e lo sviluppo di formulazioni di prodotti senza uova e latticini. Il progetto si sviluppa utilizzando come riferimento la crema a base di mascarpone impiegata come ingrediente principale nel tipico dolce italiano, il tiramisù.

Keywords: frozen dairy desserts, mascarpone, water phase stabilisers, saccharides, colloidal properties, rheology, hydrocolloids

1. Introduction

The PhD project aims to develop new formulations for milk-based frozen dessert products with higher quality, physical stability, and nutritional and health benefits. The project includes the following steps:
T1. Understanding the physical, physicochemical, and colloidal properties of mascarpone and corresponding mascarpone-based dessert custard cream, freshly made and in the frozen state.
T2. Effect of saccharides and hydrocolloids on the quality properties and stability of dairy custard cream in frozen state
T3. Design and development of dairy-free, egg-free custard cream for formulated frozen desserts.

2. Frozen desserts and sugar substitutes

The frozen dessert sector is a niche, but growing, market of the ready-to-eat products with a high level of diversification (e.g., preparations, serving or consumption temperatures, recipes, channels sales) and services,. The production process includes, after formulation, a freezing, or deep-freezing, phase and, for the consumption of some products, defrosting at chilling and/or serving temperature to obtain a soft product. The formulation of these products varies by including ingredients of various nutritional value, nature and type that, overall, contribute from one side to products with high energetic value, and, on the other, to develop simple-to-complex colloidal or dispersed systems, including foams, oil-in water emulsions and weak gels.
Frozen desserts, including ice-creams, are characterised by a stability that depends on the liquid-solid transition of the water of the aqueous phase, the ice crystals formation and the maintenance of temperatures below the freezing point during storage. However, external temperature fluctuations may induce melting and recrystallization, ice crystals size growth, and migrating crystallization along with protein aggregation, creaming or water separation, and solute crystals formation that impair the sensory quality attributes, structural properties and rheology. This occurs in particular when the products are stored at temperatures above the glass transition temperature of the maximally concentrated solution (T'g), i.e. in rubbery state. Formulation and process strategies in frozen products to enhance stability include: i) use of solutes or stabilizers with "water binding capacity" (e.g. saccharides, fibers, hydrocolloid(Tsai et al., 2020; VanWees et al., 2022) s); ii) improvement of the water holding/binding capacity of ingredients by physical technologies (e.g. high dynamic pressure for

proteins, ball milling for starch) (Ahmad et al., 2020; Sim et al., 2021).
To enhance both the nutritional value impaired by a composition rich in sugars and lipids and their safety due to allergenic ingredients (eg milk and derivatives, eggs), reformulation strategies with alternative protein sources (e.g. soy, peas) mimicking the functionality of the milk and eggs ones, sugar reduction strategies that could reduce the energy intake while keeping the frozen food stability could be interesting strategies to be investigated. The most applied formulation strategies to reduce the energetic content of frozen desserts is to find sugar replacer able to exhibit the same technological functionalities. In fact, sugars and simple saccharides serve multiple functions in foods beyond its sweetness, by acting as a bulking agent, increasing viscosity, and, thanks to their water binding capacity by reducing the water activity and the amount of freezable water. Inulin (I) is a polysaccharide whose usage in formulated foods has grown in the last years due to its prebiotic function by promoting the beneficial micro-flora in the gut (Van Loo, 2004). Moreover, the short-chain inulin enhances the sweetness of sucrose (De Castro et al., 2009) making it a good sucrose replacer and a low-calorie bulking agent (Shoaib et al., 2016). Maltodextrins (MD), partially hydrolysed starch derivatives are soluble carbohydrates whose functionalities vary significantly depending on the degree of depolymerisation (DE). MD at low DE can form weak gels and are used as texture modifiers and bulking agents replacing fat and sugar, in combination with acaloric sweeteners (Khan et al., 2018). Trehalose (T) is a disaccharide with unique technological molecular and technological functionalities related to its thermo- and cryo-stabilising effects on proteins and other biomolecules, inhibition of lipid oxidation, inhibition of starch retrogradation (Zhang et al., 2017).
The plant-based food industry, in recent years, has seen a quick development branching into almost all the fields of the food market that is progressively investing also the dairy-based frozen desserts (Kyriakopoulou et al., 2021). Increasing are the innovative and alternative products made by plant-based derivatives (e.g. soya derivatives, almond milk, vegetable oils, etc.) that are matching the needs of foods for specific consumers (vegan, lactose-intolerant, etc.) The substitution of animal-origin compounds in food formulations to obtain good products from a nutritional and sensory point of view is still challenging, due to the different characteristics of plant-origin fats and proteins. In the first case, the main feature is related to the quality of the crystals, while in the second, it is a effects of all the functionalities of the molecules in the food matrix (e.g., emulsifying, foaming, gelling ability) (Day et al., 2022).

3. Experimental Procedure

T1: Study of the physical (colour, rheology, dispersed state of the dispersed phase), microstructural and thermal properties of mascarpone cheese (two different commercial products, M1 and M2) and two corresponding custards creams (D1, D2) were made at a laboratory scale according to a standard recipe (mascarpone cheese 61.3%; fresh egg white 17.2 %; fresh egg yolk 9.2 %; sucrose 12.3 %). M samples were analyzed fresh or freshly made (t0) and D samples after 30 days of frozen storage (t30).

T2: Experiments were carried out on a model custard cream made of whole milk, egg yolk, starch, and sucrose prepared by using a Thermomixer, heated at 90°C for 7.5 min (CON). The experimental samples were prepared by substituting 25 %, 50 %, and 75 % sucrose with inulin (I), medium-DE maltodextrin (M), or trehalose (T). Custards were characterised by chemical, physicochemical, physical, and microstructural properties. Samples were analyzed just after preparation and after 30 days of frozen storage (t30). Formulations of the custards are reported in Table 2.

	Ingredient (% w/w)						
	Whole milk	**Rice starch**	**Egg yolk**	**Sucrose**	**Trehalose**	**Inulin**	**Maltodextrin**
CON	75	4	7.5	13.5	0	0	0
I03	75	4	7.5	10.13	0	3.37	0
I06	75	4	7.5	6.75	0	6.75	0
I10	75	4	7.5	3.37	0	10.13	0
M03	75	4	7.5	10.13	0	0	3.37
M06	75	4	7.5	6.75	0	0	6.75
M10	75	4	7.5	3.37	0	0	10.13
T03	75	4	7.5	10.13	3.37	0	0
T06	75	4	7.5	6.75	6.75	0	0
T10	75	4	7.5	3.37	10.13	0	0

Table 1 *Formulation of custard model samples: control (CON), and Inulin (I), Maltodextrins (M), and Trehalose (T) at different degrees of sugar substitution, 03 (25%), 06 (50%), 10 (75%)*

T3: The model cream custard (see T2) was used as control to design and formulate a plant-based model, in

which milk and egg yolk are substituted by coconut oil and plant-based proteins.

4. Methodologies

The moisture content was determined by gravimetric method; pH and a_w by a 3510 Jenway pH meter and an Aqua Lab 4TE a_w-meter, respectively. Colour was analysed with a colorimeter (CR-5 Konica Minolta) by using the CIE-LAB space: L*, a* and b* parameters were considered and used to determine the C*, and h° parameters, the ΔE and the Yellow Index (YI). Differential Scanning Calorimetry (Perkin Elmer DSC 8500) was applied to determine thermal properties including: T'g, ice and fat melting, with temperature scans and annealing approaches in the temperature range from -80°C and 70°C (10°C/min). Rheological analyses (Anton Paar MCR 302) were performed and oscillatory test (frequency strain, frequency sweep, 0.1 to 10Hz) and flow behaviour (from 0.1 to 100s^{-1}) were carried out. Confocal laser scanning microscopy (CLSM) was used to evaluate microstructure, utilising Nile red and Fast Green FCF for fats and protein staining, respectively. Along with microscopy, dispersed state (fat particle size and distribution curves) was evaluated (Mastersizer Hydro 3000, Malvern instruments); the refractive index was 1.46 (Ningtyas et al., 2019).

5. Results and Discussion

5.1 T1 Characterisation of physico-chemical, microstructural and rheological properties of mascarpone and corresponding custard dessert creams and stability over frozen state

M1 and M2 samples presented significant differences in moisture, physicochemical and rheological properties, index of two different process conditions used for their production; these properties affected also those of the corresponding dairy dessert creams (D1, D2, respectively) (Ciancetta et al., 2022). In particular the rheological properties of the D1 and D2 were significant lower G' in respect to the corresponding M1 and M2 due to the effect of the other ingredients while tanδ resulted the same for D1 and D2(data not shown). By thermal analysis the first- (fat crystallization and melting, water solid-liquid transition) and second-order (glass transition temperatures Tg and T'g) of the M and D samples were studied. A single fat crystallization peak and two melting peaks were observed; differences were observed only in the enthalpy between the M and D samples but no within each product type. D samples showed a significant increase of the glass transition onset temperature (Tg), along with a significant decrease of the freezable water content due to the effects of the solutes. The size of the fat globules of M samples was similar with D[4,3] values lower than 10µm when dissolved in SDS solution. The D[4,3] values of the D1 and D2 were significantly higher than the corresponding mascarpone samples due to the presence of egg's fats, with a flocculation index higher than 50% (higher for D1).

Freezing and frozen state (for 30 days) affected the rheological properties of both D samples with a decrease in tanδ values after 30 days of frozen storage for both samples even if of different entity, likely due to protein aggregation and degradation induced by the frozen state No significant differences on the particle size distribution and DSC analysis were observed. By CLSM images it is possible to notice a clear difference in the microstructure of M samples: M1 shows very lower and spread fat particles, while in M2 they seem to be more aggregated. Moreover, D samples' fat dispersion is in line with the related Mascarpone cheese, with D1 showing an overall lower dimension and higher dispersion. The protein structure seems to be different, since in M samples blocks of protein and fat structures are shown, while in D samples it is also possible to notice zones in which the 2 molecules are separated.

5.2 T2. Effect of saccharides and hydrocolloids on the quality properties and stability of dairy custard cream in frozen state

5.2.1 Chemical and physico-chemical properties

Overall, control and experimental samples made with different content of inulin, trehalose, and maltodextrins didn't show significant moisture content difference, ranging from 69.12±0.1 for the T03 sample and 70.35±0.01 for the M10 sample while a significant, while limited, increase of pH was observed, from 6.44±0.01 (C) to 6.54±0.01 (I), 6.66±0.01 (M) and 6.69±0.01 (T). a_w values didn't show meaningful changes with respect to the C sample, even if the substitution of S with M at the highest ratio (75%) caused an increase of the a_w due to the lower binding capacity of this component with respect to sucrose.

5.2.2 Colour

All the experimental samples showed, in respect to the control one, an increase of L* and hue angle, h,° along with a decrease of C* (*data not shown*) and YI with an effect non-concentration dependent.

Overall, due to the different L* and chromatic parameters values, a colour change, computed as ΔE higher than 3.0, reaching values higher than 5 for M and T samples was observed; these result indicates the possibility for a consumer to perceive the difference at sight. Colour is resulting from various compositional, physicochemical and structural factors that affect light reflectance onto the sample surface and, thus, the observed differences may reflect the properties of the matrix induced by the different saccharides.

5.2.3 Thermal properties

Thermal analysis was used to determine the effect of the different sucrose-substitutes on thermal properties, i.e., T'g, and ice melting enthalpy (Figure 1). The CON sample showed a T'g equal to -33°C, and the substitution of

sucrose with the alternative saccharides determined an increase of the Tg_{onset} , mainly for M samples, reaching ca. -21°C in the case of 75% of sucrose substitution; At the highest concentrations, both I and M samples showed an increase of ice melting onset temperature, while no significant differences were seen for T samples. The enthalpy of the ice melting (ΔH) resulted to increase in the M samples in respect to CON with a concentration-dependence, due to the lower binding capacity in respect to sucrose, while the opposite has been determined in the T samples. I sample don't show a concentration-dependent trend and the decrease is similar for all concentrations tested.

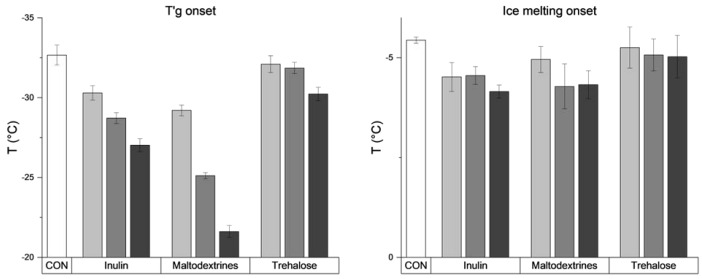

Figure 1 *Onset temperatures of T'g and ice melting of control (white) and I, M, and T samples (grey) at 25% (light grey), 50% (medium grey), and 75% (dark grey) sucrose substitution.*

5.2.4 Rheology

The substitution of sucrose with I, M and T effected the rheology of the experimental custard cream samples. In particular, I caused a significant decrease of the loss modulus (G'') with a correspondent increase of the G'' that determined a significant increase of tanδ (Figure 4). An opposite effect was determined by M and T with an increase of G' concentration-dependent effect but overall the tanδ did not change significantly due to an opposite effect on G''. The effect of the sucrose substitutes on the rheology of the custards could be referred to various factors including the different water binding capacity (see Figure 2), and/or interactions between the different solutes in the system and/or competitive effects during the formation of the structure. The apparent viscosity values are in line with G' ones, and a very similar trend can be seen. Moreover, from Herschel-Barkley regression, it is possible to notice an increase of n value for I samples, while for M samples an initial increase is seen at 25% substitution, while at 75% substitution a significant decrease is shown.

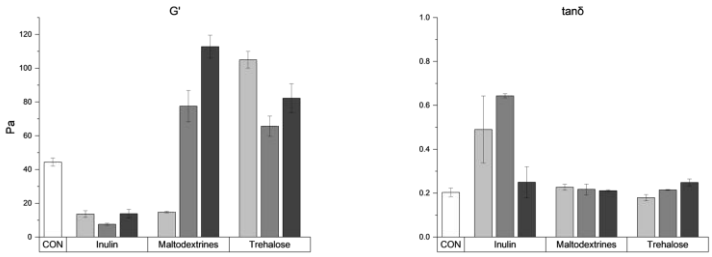

Figure 2 *G' and tanδ values of control (white) and I, M, and T samples (grey) at 25% (light grey), 50% (medium grey), and 75% (dark grey) sucrose substitution.*

5.2.5 Dispersion state

The particle size analysis was affected by the sucrose substitution with effects due to the saccharide type and its concentration. In all cases the D[4,3] values were higher with respect to CON (data not shown), with minor variations only in the case of the T samples. On the contrary, for M and I samples the D[4,3] values showed a significant increase, with an effect of the % of sucrose substitution, being higher for the higher concentrations of the polysaccharides present in the formulations . In particular, for I samples values ranged from 100% to 150% increase, for M ones from 50% to 150%, and for T samples from 50% to 80%. These results reflect the different distribution curve of the CON and experimental samples. CON exhibited a bi-modal curve, showing two peaks of µ1 and µ10 order. For T samples a decrease of the 1µm peak was seen, while for M and I samples the higher increase of D[4,3] values is related to the appearance of a second shoulder or peak, seen at the highest % of sucrose substitution at ca. 100µm order.

5.2.6 Effect of frozen storage

No significant differences have been seen for all the parameters considered, but the rheological properties with differences depending on the saccharide type and % of sugar substitution: M samples showed a significant ($p<0.05$) decrease of G' with respect to corresponding fresh samples and the effect was related to the degree of substitution. T resulted to protect the matrix after thawing since no weakening was seen at 50% and 75% substitution, while a clear weakening was showed at 25% substitution. Eventually, I samples were not analysed

due to the very high degree of syneresis.

5.3 Design and development of dairy-free, egg-free custard cream for formulated frozen desserts

Starting from a typical custard-like matrix as a control sample, a formulation design approach has been developed in order to substitute animal-origin ingredients with plant-based fats (coconut oil) and proteins. Preliminary experiments showed that the type and the concentration of protein in the matrix highly affect the viscoelastic characteristics of the samples (*data not reported*). The dispersion degree of the lipidic phase, despite the different types of fat used (coconut oil), didn't show significant differences, while at the highest concentrations of proteins, it is possible to see an increase in D[4,3] values.

6. Conclusions and Future Perspectives

T1 This research step allowed to assess the influence of Mascarpone cheese on the structure of the dairy-based custard cream, showing a strong influence on the dispersion state and rheological characteristics. On the contrary, the formulation and in particular the sugar affected the thermal properties of the aqueous phase (water crystallization and melting behaviour, T'g).

T2 The role of sugar in the physical, rheological and thermal properties of the custard matrix and was evaluated along with the effects of sucrose-substitutes with different molecular and technological functionalities. In the freshly made products, Maltodextrin and Trehalose determined an increase of the viscosity and G' that was not preserved after freezing and frozen storage in both cases, despite their different ability to affect the bind water and affect the mobility of the water phase.

Further studies are necessary to unravel the factors that could contribute to improve the stability of fresh and frozen desserts, also by to use of stabilisers (e.g. hydrocolloids) and/or innovative saccharides (e.g. modified starches).

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Structuring Oil for Healthy and Sustainable Diets: the Case Study of the Dried Template Approach

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The aim of this Ph.D. thesis is to develop innovative and environmentally-friendly methods for structuring liquid oils into soli-like materials, called oleogels. These materials are intended to be used as an alternative to saturated fats and/or as a functional ingredient for delivering health functionalities. To this aim, the feasibility of different structuring strategies has been explored. Hereafter, the strategy based on the exploitation of porous dried templates made of cellulose as an oil structurant was investigated.

Strutturazione di olio per diete sane e sostenibili: il caso studio del "*dried template approach*"

Lo scopo di questa tesi di dottorato è quello di sviluppare metodi innovativi ed ecocompatibili per strutturare oli liquidi in sostanze pseudoplastiche, note come oleogel. Questi materiali sono destinati ad essere utilizzati come alternativa ai grassi saturi e/o come ingredienti con proprietà salutistiche. A tal fine, diverse sono state le strategie studiate in questa tesi di dottorato. Qui di seguito verranno descritti i risultati relativi all'utilizzo di materiali disidratati porosi a base di cellulosa come agenti per la strutturazione dell'olio.

Keywords: oleogel, cellulose, cryogel, aerogel, particles

1. Introduction

Oleogelation can be defined as a process able to turn liquid oils into solid-like materials by exploiting the structuring properties of selected molecules, called oleogelators. Oleogels have been initially studied and developed as a feasible alternative to fats rich in saturated or trans fatty acids (*e.g.*, animal fats, tropical oils, margarine, and shortenings) due to their good capability to mimic fat technological functionalities (Patel et al., 2020). However, the interest is today further increasing due to their possible health functionalities (Calligaris et al., 2020). Currently, two main approaches for oil structuring have been proposed in the literature. Direct methods involve the use of liposoluble molecules capable of forming three-dimension networks entrapping oil upon heating and further cooling. Several components, such as saturated monoglycerides, waxes, phytosterols, ethylcellulose, and chitin, have demonstrated good oleogelation capability. This technique is considered the simplest way to structure liquid oils, despite the drawbacks related to the heating process applied, potentially affecting oil stability, as well as some limitations associated with the current EU Regulation on food additives (Patel et al., 2014). On the other side, indirect methods rely on the exploitation of a structuring network made of hydrophilic molecules. This approach enables the use of widely consumer-accepted polymeric molecules, such as polysaccharides and proteins (Patel, 2020). Among the possible approaches categorized under the indirect methods, the dry template approach is one of the most promising. It consists of the formation of a hydrogel, which is further dried. The resulting scaffold is converted into an oleogel by allowing oil absorption (De Vries et al., 2015). The structure and the functionalities of this scaffold strictly depend on the drying technique applied (Manzocco et al., 2021). In general terms, it is expected that air-drying induces a strong network collapse leading to a material with low porosity (xerogel), whereas freeze-drying (cryogel) and supercritical-CO_2-drying (aerogel) are techniques able to reduce collapse generating highly porous structures able to absorb oils (Buchtová & Budtova, 2016). In this PhD thesis, both direct and indirect methodologies to form oleogels have been investigated to quantitatively understand the structure-function relationships. Monoglycerides, waxes, and phytosterols were used to structure extra virgin olive oil (Ciuffarin et al., 2023), and the capability of the resulting oleogels to modulate lipolysis and polyphenol bioaccessibility was studied (Ciuffarin et al., *submitted*). Additionally, indirect methods have been applied by considering whey proteins and cellulose as potential oleogelators. In this paper, the feasibility of using a cellulose-based porous template for oleogel preparation will be described. Cellulose is a particularly attractive biopolymer being the most abundant polysaccharide on the Earth, not expensive and obtainable from agro-industrial vegetable side

streams, in a closed loop that avoids the generation of large quantities of waste (Pires et al., 2022). In particular, monoliths of cryogels and aerogels were prepared by freeze-drying (FD) or supercritical-CO_2-drying (SCD) of cellulose hydrogels and characterized for their structural features (SEM microstructure, BET-specific surface area, porosity, pore volume, firmness, density and interaction with oil). In the last part of the work, to modulate oleogel functionalities, cryogel particles were considered instead of monoliths. The obtained results open interesting novel possibilities in using renewable cellulose materials for oil structuring.

2. Materials and Methods

2.1 Preparation of cellulose hydrogels, cryogel, and aerogel monoliths and particles

Cellulose hydrogels were prepared as described by Ciuffarin et al. (2023) starting from microcrystalline cellulose (Avicel®, pH-101, Sigma Aldrich). Briefly, cellulose was dispersed at -5 °C in an 8% NaOH-water solution to obtain a final concentration of 3, 4, and 5 % (w/w). Around 6 mL of the solution was poured into cylindrical polypropylene vials (2.7 cm in diameter) and heated at 50 °C for 2 h to allow gelling. The samples were finally washed until a pH of 7.0 was reached. The cellulose hydrogels were freeze-dried (Cryotec Cosmos, Saint-Gély-du-Fesc, France) to obtain cryogels; or dried with supercritical CO_2 (Homemade set-up of PERSEE Mines Paris, France) after a phase of solvent substitution (water with ethanol) to obtain aerogels (Ciuffarin et al., 2023).

For cryogel particle preparation, 5% (w/w) cellulose hydrogel monoliths were ground in a ratio of 2:1 with deionized water using a high-speed homogenizer (DI 25 Basic, IKA Werke, Staufen im Breisgau, Germany) at 14,000 rpm for 3 min. The obtained viscous solution was placed in aluminum containers and freeze-dried (72 h, -80 °C, 10 mTorr). Since cryogel particles presented uneven size (56.4% < 100 µm, 39.8% in the range of 100-500 µm, and 1.3% > 500 µm), they were sieved, and only particles with dimensions lower than 100 µm were collected and used to prepare oleogels. Dried monoliths and particles were stored in desiccators containing granular silica gel at room temperature until analysis.

2.2 Oleogel preparation

After preliminary trials, here not described, the best preparation conditions needed to obtain a self-standing material with no visible oil release were defined by mixing sunflower oil and cryogel particles. In particular, cellulose cryogel particles were manually mixed with sunflower oil at a 1:2.4 ratio (w/w).

2.3 Structural characterization of cryogel and aerogel monoliths and particles

Macroscopic images. Sample images were acquired using an image acquisition cabinet and a Google Pixel 6 (Alphabet, Mountain View, California, USA). The light was provided by a LED strip properly placed to minimize shadow and glare.

Microstructure. SEM micrographs were obtained using a MAIA-3 (Tescan, Brno, Czech Republic), equipped with detectors of secondary and back-scattered electrons. The internal cross-section of the samples was coated with a 14 nm layer of platinum with a Quorum Q150T metallizer (Quorum Technologies, East Sussex, UK) to prevent the accumulation of electrostatic charges and image defaults. The observations were performed with an acceleration voltage of 3 kV.

Volume. Monolith volume was measured by a CD-15APXR digital caliper. Volume variation (ΔV, %) during the conversion of hydrogels to cryogels or aerogels was measured.

Density, porosity, pore volume. Monolith envelope density ($\rho_{envelope}$) was measured using the Micromeritics GeoPyc 1360 Envelope Density Analyzer (Norcross, Georgia, USA) with the DryFlo® powder as a fluid medium. Each sample was measured in 5 cycles with an applied force of 27 N. Porosity (eq. 1) and pore volume (eq. 2) were calculated from the envelope ($\rho_{envelope}$) and cellulose skeletal density ($\rho_{skeletal}$ = 1.5 g cm^{-3}, (Sun, 2005)):

$$Porosity\ (\%) = \left(1 - \frac{\rho_{envelope}}{\rho_{skeletal}}\right) \cdot 100 \qquad \text{(eq. 1)}$$

$$Pore\ volume\ (cm^3 g^{-1}) = \frac{1}{\rho_{envelope}} - \frac{1}{\rho_{skeletal}} \qquad \text{(eq. 2)}$$

Specific surface area. Monolith-specific surface area (S_{BET}) was determined by measuring N_2-adsorption isotherm at 77 K with the Micromeritics ASAP 2020 (Norcross, Georgia, USA) and using Brunauer, Emmett, and Teller (BET) approach (Brunauer et al., 1938). Prior to measurements, samples were degassed for 5 h at 70 °C.

Cryogel particle density. The density (g cm^{-3}) of cryogel particles and microcrystalline cellulose (control) was measured by weighing 1 mL of dried material in a graded cylinder.

Cryogel particle size. Particle size was measured by using a vibrating sieve equipped with 2 different sieves (100, and 500 µm). The separated fraction was weighted and the distribution, over the total weight, was evaluated.

2.4 Monoliths interaction with oil

Monoliths oil absorption kinetics. Monoliths were cut into 1 cm^3 cubes volume, weighted (W_0), and immersed into Petri plates containing sunflower oil at room temperature. At defined time intervals, samples were withdrawn, wiped with absorbent paper, and weighed (W_t). The experiment was carried out until a constant weight was reached (*plateau* or equilibrium value), as indicated by no weight variation in 3 consecutive measures. Absorbed oil at each time was expressed as the ratio between weight gain at time t (min) and the initial weight of the cryogel or aerogel sample (eq. 3).

$$Absorbed\ oil\ \left(g_{oil}/g_{dry\ matter}\right) = \frac{(W_t - W_0)}{W_0} \qquad \text{(eq. 3)}$$

The maximum oil absorption capacity was taken at equilibrium.

Oil holding capacity. At monoliths absorption equilibrium, around 100-200 mg of sample (W_1) was placed into 1.5 mL microtubes and centrifuged at 15,000 ×g for 30 min (Mikro 120, Hettich Zentrifugen, Andreas Hettich GmbH and Co, Tuttlingen, Germany). After centrifugation, the released oil was accurately wiped using absorbing paper and the sample was weighted again (W_2). Oil holding capacity (OHC) was calculated as the percentage ratio between the weight of oil retained in the sample after centrifugation and the total oil weight initially present (eq. 4).

$$Oil\ Holding\ Capacity\ (\%) = \frac{S \cdot W_1 - (W_1 - W_2)}{S \cdot W_1} \cdot 100 \qquad \text{(eq. 4)}$$

where S represents the weight fraction (%) of the oil initially present in the sample. The same centrifugation procedure was performed for the oleogel sample.

2.5 Oleogel characterization

Confocal microscopy. A 0.2 % Nile Red aqueous solution and 0.01% of Fluorescent Brightener 28 were used to stain the oil and the cellulose, respectively, by gently hand-mixing the oleogel samples. Cryogel particles were placed on the microscope slide, covered with a cover slip, and observed at 100× magnification (Leica TCS SP8 X confocal system, Leica Microsystems, Wetzlar, Germany). Images were elaborated using the software LasX 3.5.5.

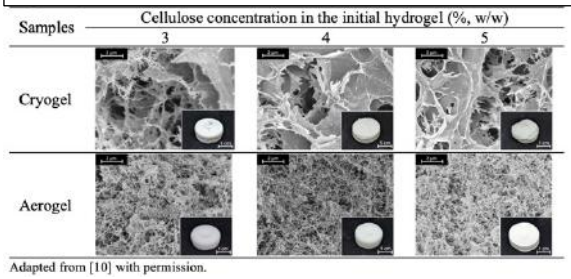
Firmness. Firmness was evaluated using an Instron 4301 (Instron LTD., High Wycombe, UK) with a 6.2 mm diameter cylindrical probe and a 1 kN compression head. Monoliths and oleogel samples were compressed at a crosshead speed of 25 mm min^{-1}, and the maximum force (N) needed to penetrate the sample by 2 mm was measured.

Rheological properties. Oleogel viscoelastic properties were tested using an RS6000 Rheometer (Thermo Scientific RheoStress, Haake, Germany), equipped with a Peltier system. Measures were performed using a parallel plate geometry at 20 °C with a gap of 2.0 mm. Oscillatory sweep tests to identify the linear viscoelastic region (LVR) were performed increasing stress from 1.0 to 1.0 × 10^4 Pa at 1 Hz frequency. Critical stress (Pa) was identified as the stress value corresponding to a 10% drop in G′ value. Frequency sweep tests were then performed increasing frequency from 0.1 to 10 Hz at stress values selected in the LVR and G' – G" recorded at 1 Hz.

2.6 Statistical analysis

Data were obtained by at least triplicate measurements. Data were reported as mean ± standard deviation and subjected to one-way analysis of variance (ANOVA) and Tukey's Honest Significant Differences test ($p < 0.05$) using R for Windows (The R foundation for statistical computing).

Table 1. *Visual appearance and SEM microstructure of cryogel and aerogels prepared from cellulose solution containing 3, 4, 5% (w/w) cellulose.* [a-e]*: values in different letters are statistically different (p<0.05).*



Adapted from [10] with permission.

3. Results and Discussion

3.1 Cryogel and Aerogel Monolith Characterization

The visual appearance and the microstructure of cryogel and aerogel monoliths are presented in Table 1. Cryogels were visually opaque with evident cracking, while aerogels appeared more homogeneous. These results were associated with the microstructural feature: cryogels had larger pores with flat walls in comparison to aerogels showing a fibrillated network with smaller pores. The conversion of hydrogels into cryogels resulted in a slight increase in volume (5 – 10%), attributed to ice crystal growth during freeze-drying. In contrast, aerogel preparation caused a volume contraction (≈ 23%), likely due to differences in solubility parameters between cellulose, ethanol, and CO_2. Consequently, cryogels had lower density (0.056 – 0.077 g cm^{-3}) than aerogels (0.077 – 0.112 g cm^{-3}) prepared from cellulose solutions of the same concentration, while higher cellulose concentrations led to denser cryogels and aerogels. Both cryogels and aerogels exhibited high porosity (>

90%), with slightly lower porosity observed in aerogels. The specific surface area (S_{BET}), which indicates the presence of mesopores and small macropores (< 200 nm), was more than 10 times higher for aerogels (~ 380 $m^2 g^{-1}$) compared to cryogels (~ 30 $m^2 g^{-1}$). This observation agrees with SEM analysis. Due to these characteristics, cryogels exhibited lower firmness compared to aerogels (Figure 1) due to the weaker structure caused by the FD ice crystal growth.

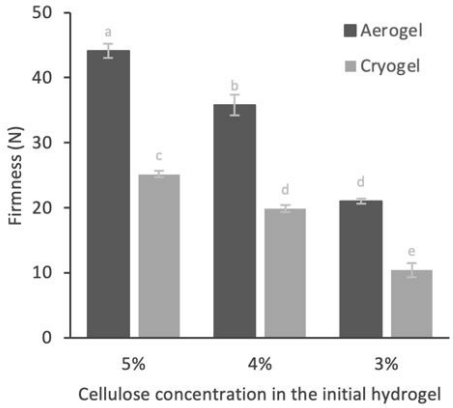


Figure 1. *Firmness of cryogel and aerogels prepared from cellulose solution containing 3, 4, 5% (w/w) cellulose. [a-e]: values in different letters are statistically different (p<0.05).*

3.2 Interaction with oil

The potential use of cellulose cryogels and aerogels as innovative fat replacers requires understanding their interaction with oil. The kinetics of oil absorption by cryogels and aerogels is reported in Figure 2A. Cryogels exhibited faster oil absorption compared to aerogels, reaching the equilibrium within 15-60 minutes, while aerogels showed more gradual absorption kinetics (200-250 minutes). These variations in absorption kinetics can be attributed to the different morphology of cryogels and aerogels, with aerogels having smaller pores compared to cryogels.

In Figure 2B, the maximum oil absorption values of the materials are plotted against the material pore volume. The absorption values ranged from 4 to 8 $g_{oil}/g_{dry\ matter}$ for aerogels and 8 to 13 $g_{oil}/g_{dry\ matter}$ for cryogels, reflecting the different pore volumes of the materials. This highlights the significant role of material pore volume in determining fluid uptake at equilibrium, in fact, an $R^2 = 0.96$ linear correlation was found. Regarding their ability to absorb oil and retain it, OHC was tested and excellent results were obtained (> 96%), regardless of their morphological feature.

These results demonstrate that cellulose aerogels and cryogels can absorb substantial amounts of oil with interesting potentialities for food applications. For this reason, further experiments were performed to improve the plasticity of the resulting material.

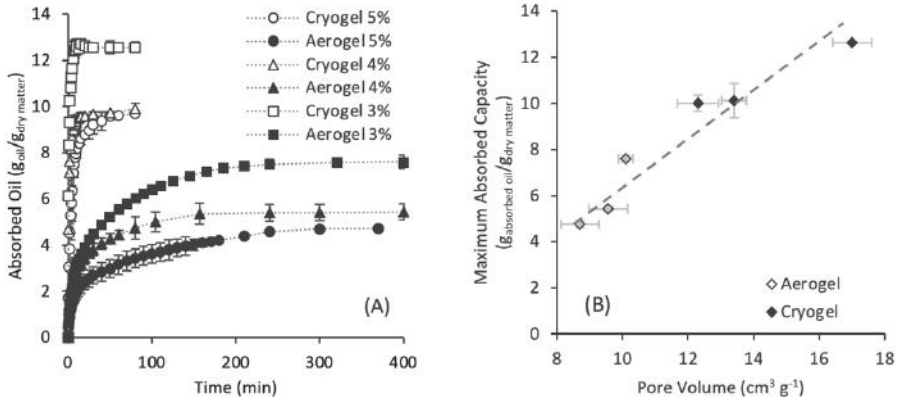
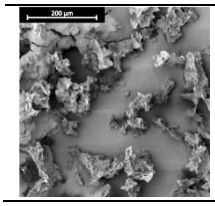
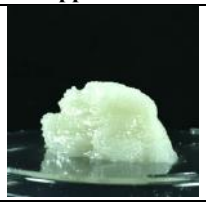
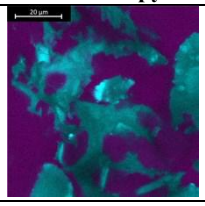


Figure 2. *(A) oil absorbing kinetics and (B) maximum oil absorption capacity of cryogel and aerogel prepared from hydrogels containing 3, 4, 5% (w/w) cellulose. Dashed line in (B) is the least square approximation with R2 = 0.96. Adapted from Ciuffarin et al (2023) with permission.*

3.3 Cellulose-based oleogel preparation and characterization

Despite the good oil absorption capability, monoliths resulted in hard materials not applicable to mimic the functionalities of plastic fat. Thus, cryogel monoliths were ground before drying to obtain fine particles that potentially could absorb oil and generate a plastic material. Cryogel particle microstructure and oleogel characterization results are reported in Table 2. As visible from SEM images (Table 2), cellulose particles presented an uneven fibril-like surface, similar to that observed in the cryogel monolith (Table 1), characterized by the presence of pores. The observed porosity was confirmed by the density of 0.31 g cm^{-3}, much lower than that of the microcrystalline cellulose used for their preparation (0.42 g cm^{-3}). As noticeable in Table 2, a self-standing material was obtained upon oil addition, showing a firmness value of 4.99 N and gel-like behavior being G' > G". Moreover, viscoelastic properties were comparable to those of traditional plastic fats in terms of critical stress and G' values (Patel et al., 2020). As outlined in the study by Plazzotta et al. (2020), the ability of dried templates to structure oil can be attributed to two key mechanisms:

Table 2. *SEM of cellulose cryogels particles (from 5% cellulose hydrogel) and macroscopic appearance, confocal microscopy (blu = cellulose, pink = oil), firmness, and rheological parameters of cellulose-based oleogel.*

Particles	Oleogel				
SEM	Macroscopic Appearance	Confocal Microscopy	Firmness (N)	Critical Stress (Pa)	G' × 10^4 (Pa)
			4.99 ± 0.71	192 ± 37	203.1 ± 35.6

the pore capacity to absorb oil through capillary forces, both within its inner structure and at the surface where particles and oil interact; the formation of a biopolymer network that entraps oil in the interstitial spaces between particles, facilitating particle-particle interactions through hydrogen bonding. Confocal microscopy of cellulose-based oleogel (Table 2) confirmed the occurrence of both mechanisms, showing the embedding of the oil within pores as well as its holding in the interparticle spaces.

4. Conclusions and future perspectives

Results demonstrated that cellulose-based dried materials are promising oil-structuring templates. The drying technique applied to obtain the templates was found to significantly influence the morphology of the porous material, affecting the oil absorption and entrapping capacity, with cryogel being the best-performing material. Upon cryogel grinding before the drying step and further oil absorption, a self-standing oleogel with mechanical and rheological properties comparable to those of plastic fats can be obtained. These results open interesting opportunities to exploit cellulose as oil structuring material in food formulations with reduced saturated fat content. Future studies could better clarify the potentialities of cellulose-based oleogels for different food applications.

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Enrichment of Extra Virgin Olive Oil for the Development of Functional Oil for Special Consumers

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The aim of this PhD research project was to evaluate the impact on the shelf life evolution and bioactivity of functional olive oils obtained by EVOO enrichment with selected matrices by using two different technological approaches: malaxation and infusion. The obtained flavoured virgin olive oils (FVOOs) were found to be pleasant from a sensorial point of view and with marked health properties during shelf life.

Funzionalizzazione di un olio extravergine di oliva per la creazione di prodotti destinati a categorie speciali

Lo scopo del progetto di dottorato è stato quello di valutare l'impatto sull'evoluzione della shelf life e la bioattività di oli di oliva funzionalizzati ottenuti dall'arricchimento di EVOO con matrici selezionate, utilizzando due diversi approcci tecnologici: gramolatura e infusione. Gli oli di oliva vergini aromatizzati (FVOOs) ottenuti sono risultati gradevoli dal punto di vista sensoriale e con spiccate proprietà salutistiche durante la shelf life.

Key words: Functional olive oil; antioxidant activity, enzymatic activity; health properties.

1. Introduction

In accordance with the PhD thesis project previously described (Custureri, 2021), in this oral presentation the following aspects were analysed:

A1) EVOO enrichment with selected matrices by using two different technological approaches: malaxation and infusion;
A2) EVOO and FVOOs physical-chemical and sensorial analysis;
A3) EVOO and FVOOs UHPLC analysis;
A4) Determination of antioxidant activity by multi-target approaches and enzyme inhibitory assays related to health status;
A5) Evaluation of EVOO and a selection of FVOOs aroma profile by the optimization of a SPME-GC-MS method;
A6) Statistical analysis.

Extra-virgin olive oil (EVOO) is an essential condiment used by the population of the Mediterranean basin.
Several published studies document that most of the health effects of the Mediterranean diet can be ascribed to EVOO. In fact, consumption of EVOO is related to a reduction in the oxidation process of biomolecules such as lipids and DNA, a reduction in insulin-resistance and an improvement in the lipid profile etc. These effects protect from both metabolic disorders and cardiovascular disease (Buckland and Gonzalez, 2015). Recently, an increasing amount of scientific evidence has revealed that phenolic compounds which represent only ~2% of EVOO may also contribute to the healthy features of EVOO (Jimenez-Lopez et al., 2020). The chemical composition of the EVOO varies according to the olive cultivar, the harvesting period and geographical origin, the pedoclimatic conditions of growth (Giuffrè, 2017). Italy is the second largest EVOO producer with protected designation of origin (PDO) and protected geographical indication (PGI) mark. Many of these cultivars are located in Calabria, a region of southern Italy, due its favourable microclimate conditions (Marra et al., 2013). Among them the Ottobratica cultivar represent one of the most cultivated (Sicari at al., 2009). Spices are widely used to increase food palatability (Issaoui et al., 2016). Moreover, they provide some biological effect and extend the shelf life of food (Opara and Chohan, 2014). These actions are due to their phytochemical content of polyphenols, terpenoids and carotenoids (Wahyuni et al., 2013). The addition of herbs and spices to EVOO has become more popular in recent years, due to consumer demand of "gourmet oils" (Clodoveo et al., 2016). The addition of spices or other flavourings means the resulting oil no longer satisfies the European Union Commission definition for extra virgin olive oil, but can be defined as a Flavoured Olive Oil (FVOO). Several research papers have investigated the effect of the addition of herbs and spices on oil nutritional quality, oxidative stability, and sensorial characteristics. Clodoveo et al. (2016) compared the impact of different FVOOs production techniques (infusion of herbs into the oil, addition of herbs to the crushed olives before malaxation and application of ultrasound before the olive paste malaxation) on the quality of the FVOO.
In this context, our work examines the effect of two different technological approaches: malaxation and infusion.

The following plant matrices were used for enrichment: Turmeric (*Curcuma longa* L.), Ginger (*Zingiber officinale* R.), Mace (*Myristica fragrans* Houtt.), Spirulina (*Spirulina* sp.), Bergamot (*Citrus bergamia* Risso & Poiteau) and Goji berries (*Lycium barbatum* L.).

2. Experimental procedures

Olive fruits (*Olea europea* L.) of Ottobratica variety were harvested in Reggio Calabria province (Italy). The oil extraction was performed by a mini-pressing apparatus. The parts of plants and spices were purchased at a local supermarket, except for the bergamot fruit and goji berry puree (purchased from Calabrian producers). All the matrices were added as a powder with the exception of bergamot fruits and goji berries, which were cut into slices and used as a puree, respectively. For the addition during malaxation (1%), the extraction was performed at room temperature and malaxation lasted for 40 minutes. With the aim of making a comparative analysis, the enrichment was also done by infusion (2%). The oil was infused for 30 days in the dark and under constant agitation. The enrichment matrices were not added directly to the oil, but small bags, similar to tea bags, were created with sterile gauze. Bergamot and goji berry puree, before the addition by infusion, were freeze-dried. The additions were made with single matrix or in combination with two or three matrices to evaluate any antagonistic or synergistic effect. The obtained FVOOs were filtered and packaged in amber glass bottles with a capacity of 100 mL with threaded screw cap with drip catcher. The analyses were made for EVOO and FVOOs at different pre-established times to evaluate stability throughout one year of storage. Samples were analysed in triplicate. The analysis of variance (one-way ANOVA) was conducted by applying the post hoc Tukey test at $p < 0.05$ (SPSS software, 21.0 version, Armonk, NY, USA).

3. Results and Discussion

In this discussion, data concerning the enrichment with Turmeric (T), Mace (M) and Ginger (G), produced both by infusion (INF) and by malaxation (MAL) are examined. The unflavoured olive oil during the storage suffered a slight oxidation, but it could be considered an extra virgin olive oil (EVOO) due to the values of free acidity (FA) ranging from 0.68 to 0.84 % at T0 and T360, respectively and peroxide values (PV) from 9.45 to 17.89 mEq O_2 kg^{-1} at T0 and T360, respectively. Among the FVOOs, FA and PV naturally increased during the storage, but did not greatly affect the quality of the oils. Only T-MAL and T-INF, showed a lower level in term of FA than the EVOO, even after the storage. A fundamental parameter for consumer acceptability is colour, and the addition of spices can greatly alter this. In EVOO there was a significant decrease in chroma (C*) (from 7.24 to 2.24). For all the FVOOs, a decrease was found with values approximately four-times lower than T0 after 360 days of storage, too. The evaluation of total carotenoids content (TCC) highlighted a great variability among the FVOOs. The values of EVOO ranged from T0 6.15 to T360 4.80 mg kg^{-1}. Although the content of carotenoids is highly variable, at the end of storage all the samples had a greater content than the EVOO, except for M-MAL. The different procedures applied led to different results and the infusion allowed a major recovery and stability of these compounds (Figure 1). The total phenolic content (TPC) trends are described in Figure 2. EVOO showed a TPC value of 418.51 mg kg^{-1} and an increase in phenols was observed in all the FVOOs. The highest values were discovered in the first 30 days, probably for a solubilisation of these compounds, followed by a natural decline after 60 days. Regarding FVOOs-MAL, M at the end of storage presented the upper level of TPC (557.82 mg kg^{-1}). However, regarding the FVOOs-INF, all the samples presented higher values in comparison to the control and about two-times higher than the FVOOs-MAL. These results could be explained both by the inhibition of β-glycosidase activity and by the hydrolysis/partition phenomena toward lipid and water phases of biophenols during the malaxation and centrifugation steps (Sacchi et al. 2017). On the contrary the addition by infusion of herbs and spices to EVOO lead to FVOOs richer in TPC. The combination of the plant matrix with olive paste does not seem to be useful for the equilibrium of these molecules, presumably by the partitioning phenomena towards the lipidic and aqueous phases and by phenomena of antagonism. Among the goals of the present study there is the evaluation of the α-tocopherol content. The starter values were similar for all the FVOOs, with the exception of the mixtures with T that possessed the lowest values. At the end of storage, in all the FVOOs, the content of α-tocopherol remained higher than the control; in particular G-MAL and M-MAL showed the greatest protection against α-tocopherol loss, maintaining the highest values (101.16 and 97.21 mg kg^{-1}, respectively) (Figure 3). Ottobratica EVOO showed a promising 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity with IC_{50} values from 12.33 to 29.54 μg mL^{-1} for T0 and T360 samples, respectively. A similar situation was observed also with 2,20-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) test. A loss of antioxidant activity, in terms of protection from lipid peroxidation was observed using the β-carotene bleaching test (IC_{50} values from 48.72 to >100 μg mL^{-1} for T0 and T360 samples, respectively) (Figure 4). Data from ferric reducing antioxidant power (FRAP) assay revealed that regardless of the storage time, the EVOO results were lower than the butylated hydroxytoluene (BHT), used as positive control (from 25.01 to 4.31 μM Fe(II) g^{-1} for T0 and T360, respectively) (Figure 5).

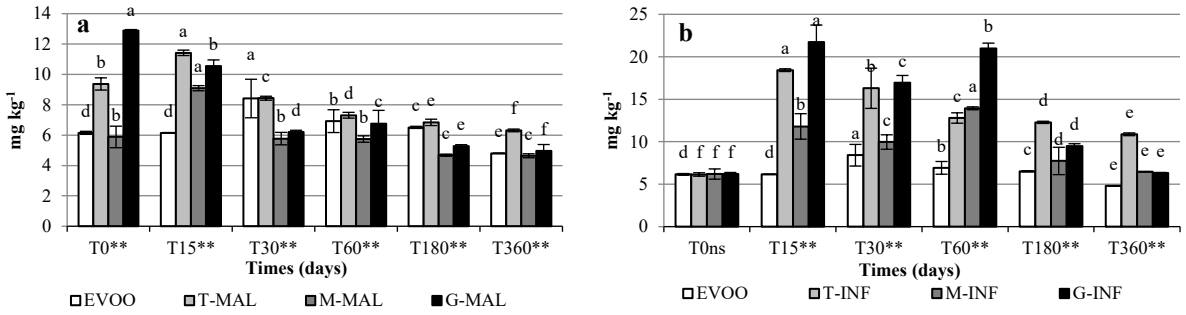

Figure 1 *Total carotenoid content of EVOO, FVOOs-MAL (**a**) and FVOOs-INF (**b**). Data are expressed as means ± S.D. (n= 3). Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: **p < 0.01. Results followed by ** differ significantly at the same time of analysis; results followed by letters differ significantly during the storage.*

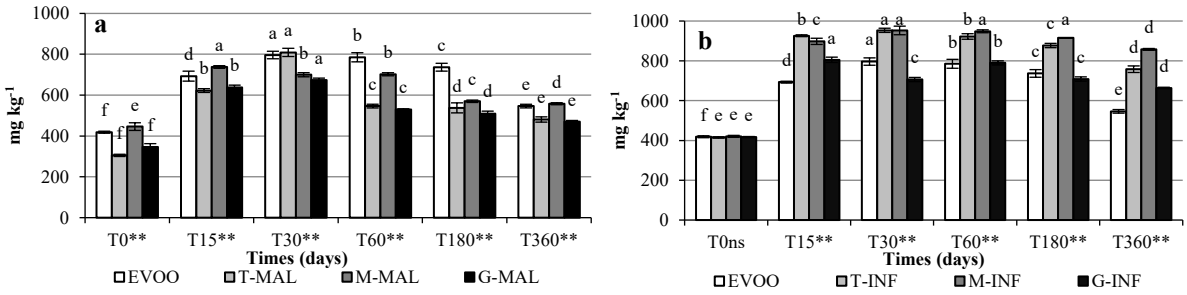

Figure 2 *Total phenolic content of EVOO, FVOOs-MAL (**a**) and FVOOs-INF (**b**). Data are expressed as means ± S.D. (n= 3). Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: **p < 0.01. Results followed by ** differ significantly at the same time of analysis; results followed by letters differ significantly during the storage.*

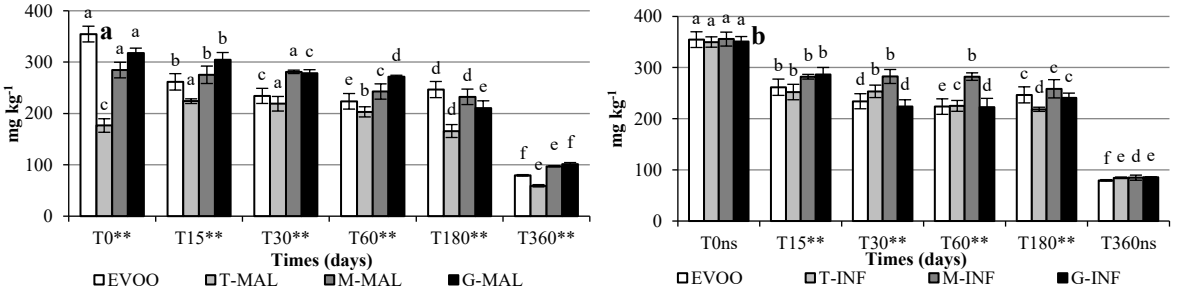

Figure 3 *α-tocopherol content of EVOO, FVOOs-MAL (**a**) and FVOOs-INF (**b**). Data are expressed as means ± S.D. (n= 3). Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: **p < 0.01. Results followed by ** differ significantly at the same time of analysis; results followed by letters differ significantly during the storage.*

The antioxidant potential of dry spice extracts showed that mace exhibited the highest DPPH radical scavenging potential (IC_{50} 16.56 μg mL^{-1}) whereas turmeric was the most active in the ABTS test (IC_{50} 3.14 μg mL^{-1}). T-MAL sample (at T0) showed a promising radical scavenging effect with IC_{50} values of 9.49 and 3.47 □g mL^{-1} for the DPPH and ABTS tests, respectively. A ferric reducing ability power better than those reported for the positive control BHT was observed only for G-MAL sample (86.42 vs 63.42 μM Fe (II) g^{-1}) (Figure 5). This biological property is positively correlated with the α-tocopherol content (r=0.97). Storage time reduces the protection from lipid peroxidation measured by the β-carotene bleaching test. Notable results were obtained with T and G in both enrichment procedures (Figure 4). In particular, in the β-carotene bleaching test, G-MAL was positively correlated with TCC (r=0.99). Moreover, in both the FRAP and β-carotene bleaching tests, after 12 months of storage G-MAL and T-MAL samples, are characterized by a good antioxidant potential, better than those found for G-INF and T-INF. The evaluation of bioactivity includes, also the inhibition of carbohydrate hydrolysing enzymes and lipase. Ottobratica EVOO exhibited IC_{50} values from 269.02 to 289.32 μg mL^{-1}, and

from 137.34 to 778.23 μg mL^{-1} at T0 and T360, for α-amylase and α-glucosidase, respectively. IC_{50} values from 143.46 and 312.97 μg mL^{-1} at T0 and T360 were recorded against lipase. From the analysis of data, it emerges that sample M is characterized by a good pancreatic lipase inhibitory activity (with IC_{50} value of 83.6 μg mL^{-1}). Promising results were obtained also with M-INF that showed IC_{50} values ranging from 62.25 to 138.66 μg mL^{-1} at T0 and T360, respectively whereas values from 63.45 to 195.96 μg mL^{-1} at T0 and T360 were recorded for G-INF. By UHPLC analysis, the trend is highly variable among the FVOOs. With regard to sensorial analysis, the FVOOs were tested by a group of expert panellists. They scored different overall acceptability and are listed below in descending order for both approaches: mace, ginger and turmeric (Figure 6 and 7).

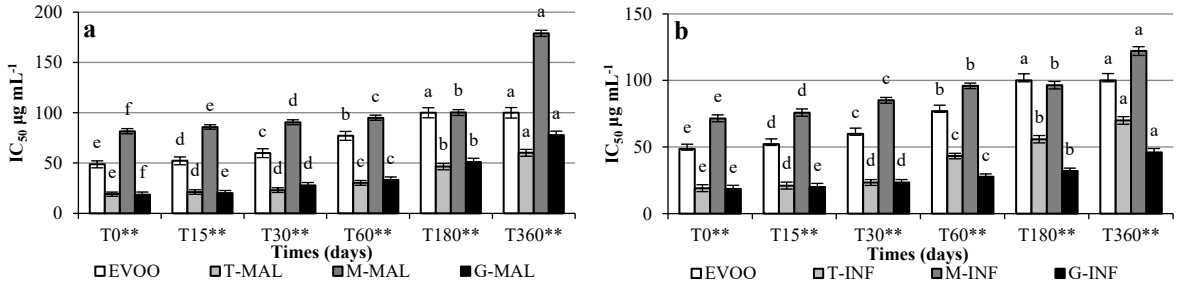

Figure 4 *β-carotene bleaching test of EVOO, FVOOs-MAL (**a**) and FVOOs-INF (**b**). Data are expressed as means ± S.D. (n= 3). Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: **p < 0.01. Results followed by ** differ significantly at the same time of analysis; results followed by letters differ significantly during the storage.*

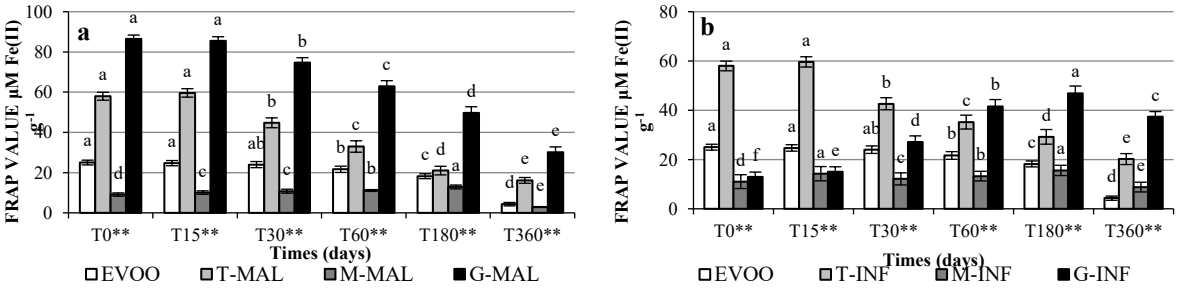

Figure 5 *FRAP assay of EVOO, FVOOs-MAL (**a**) and FVOOs-INF (b). Data are expressed as means ± S.D. (n= 3). Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: **p < 0.01. Results followed by ** differ significantly at the same time of analysis; results followed by letters differ significantly during the storage.*

The assessors were not able to identify the enrichment matrices for all the FVOOs. The EVOO was characterized by the presence of a slight "muddy" and "sludge" defects. Starting from the most appreciated, M-MAL and M-INF, a "citrusy" note appeared and there was an increment in the "vegetable" and "green fruity" notes whereas in M-MAL sample the attribute "sweet" significantly increased. In G-INF emerged a strong rise in the "pungent" note and resulted the most "spicy" FVOOs moreover the panellists asserted that in G-INF the "muddy" defect was not covered differently to G-MAL. The most characteristic note of T-MAL and T-INF was obviously the colour, which became a bright yellow. They are also characterized by a high "ripe fruity" and "spicy" attributes. They differed from each other because in T-MAL the defects of the starting oil were covered and its flavour was more balanced than T-INF, in fact T-MAL sample resulted the most sweet and equilibrated FVOOs in the gustatory sensations. To evaluate the volatile profile, FVOOs were subjected to different storages to simulate either producer's bottling conditions (with no headspace) or domestic consumption (with 50% headspace). Solid-phase microextraction followed by gas chromatography coupled with mass spectrometry (SPME GC-MS) analysis, under previously optimized conditions (T=44°C, t_{eq}=10 min, t_{ext}=60 min), showed no significant changes for most volatiles stored with no HS. However, storage under domestic conditions favoured the loss of a few volatiles, this effect being more evident for extended storage. Among the FVOOs, mace samples possessed the richest volatile profiles.

4. Conclusions and Future Perspectives

The obtained results confirmed the complexity and the variability of the EVOO aromatization process. In fact,

the addition of spices does not always improve either the shelf life of the olive oil nor its quality. Frequently the low lipophilic character of the bioactive compounds deriving from the flavouring matrices limits their transfer to the FVOO. The blend with turmeric, in infusion or in malaxation, produces FVOOs with the lowest level in free acidity. FVOOs obtained by malaxation lead to products characterized by a high and stable α-tocopherol content and a better antioxidant activity, but the infusion approach generates results higher in term of total phenolic content. FVOOs obtained by the addition of ginger and mace are characterized by a promising pancreatic lipase inhibitory activity. Concerning biological activity, infusion gives the most advantageous results. However, a significant reduction of bioactivity was observed during shelf life. The malaxation enrichment process promotes the covering of any defects in the starting olive oil; moreover, enriching an olive oil with slight defects by malaxation could be favourable thanks to a masking effect on the negative attributes, enhancing those of the spice. The FVOOs-MAL possess the richest volatile profiles. Finally, it could be stated that there is no best enrichment procedure valid for all matrices.

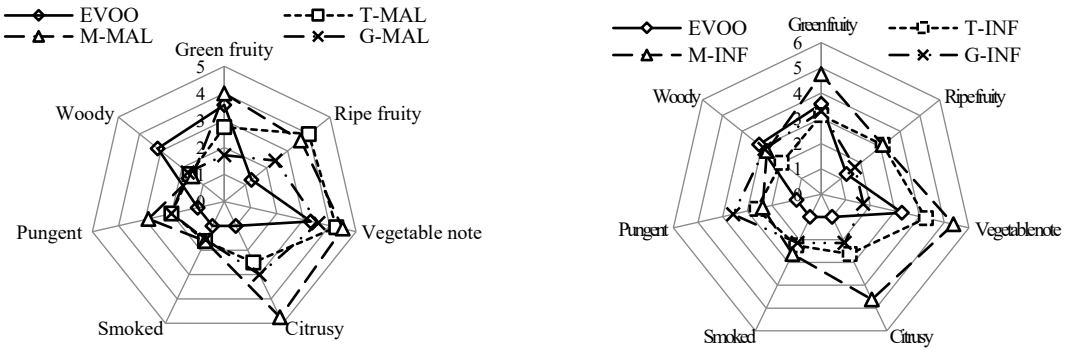

Figure 6 *Olfactory sensations of EVOO, FVOOs-MAL and FVOOs-INF.*

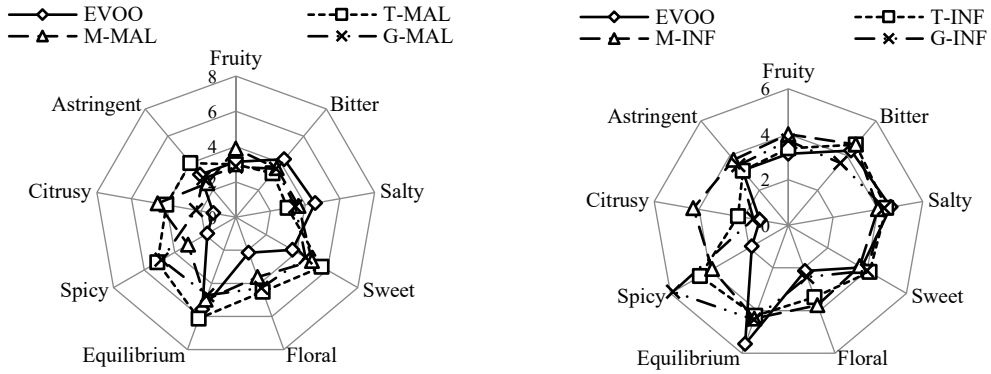

Figure 7 *Gustatory sensations of EVOO, FVOOs-MAL and FVOOs-INF.*

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Finding Nemo's family: Enhancing NIR-Based Authentication of Mediterranean Anchovies- The Influence of Spectra Pre-processing and Machine Learning Techniques

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In this study, we explored the impact of spectra pre-processing techniques and machine learning algorithms on the authentication of anchovies using near-infrared (NIR) spectroscopy. Raw NIR spectra of anchovy samples underwent various pre-processing methods, either individually or in combination. Subsequently, the spectra were analyzed using unsupervised Principal Component Analysis (PCA) followed by Linear Discriminant Analysis (LDA). The results obtained from LDA were compared to those obtained using machine learning (ML) models, specifically Support Vector Machines (SVM). SVM outperformed PCA-LDA modelling in terms of classification accuracy and did not involve any data reduction due to its superior computing ability.

Finding Nemo's family: Miglioramento dell'autenticazione basata su spettroscopia NIR delle alici del Mar Mediterraneo: l'influenza delle tecniche di pre-elaborazione degli spettri e di Machine Learning

In questo studio, abbiamo esplorato l'impatto delle tecniche di pre-elaborazione degli spettri e degli algoritmi di machine learning sull'autenticazione delle alici utilizzando la spettroscopia nel vicino infrarosso (NIR). Gli spettri NIR grezzi di campioni di alici sono stati sottoposti a vari metodi di pre-elaborazione, singolarmente o in combinazione. Successivamente, gli spettri sono stati analizzati utilizzando l'analisi delle componenti principali (PCA) non supervisionata seguita dall'analisi discriminante lineare (LDA). I risultati ottenuti dalla LDA sono stati confrontati con quelli ottenuti utilizzando modelli di machine learning (ML), nello specifico Support Vector Machines (SVM). SVM ha superato la modellazione PCA-LDA in termini di accuratezza della classificazione e non ha comportato alcuna riduzione dei dati grazie alla sua capacità di elaborazione superiore.
Key words: NIR spectroscopy, fish authentication, machine learning modelling, quality control, traceability

1. Introduction

This oral communication highlights the primary outcomes of four activities with the following objectives:
A1) Comparison of raw NIR spectra with processed spectra using various pre-processing methods, both individually and in combination, resulting in a total of nine different outputs.
A2) Evaluation of the authentication models based on Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) for each pre-processing technique employed.
A3) Assessment of the authentication model based on Support Vector Machines (SVM) using linear and polynomial kernel for each pre-processing technique utilized.
A4) Comparison of the authentication model performance between PCA-LDA and SVM approaches, determining their respective strengths and limitations.
The presentation will provide a comprehensive overview of the findings from these activities, shedding light on the effectiveness of different pre-processing methods and the performance of PCA-LDA, linear kernel SVM and polynomial kernel SVM models in NIR-based authentication.

2. Application of NIR Spectroscopy for Authentication

Food authentication is of utmost importance in ensuring product quality, safety, and preventing fraudulent practices in the food industry. Near-infrared (NIR) spectroscopy has emerged as a powerful tool for food authentication due to its non-destructive nature and ability to provide rapid and reliable results. NIR spectroscopy utilizes the interaction of light with the molecular structure of food samples to generate spectral data. These spectra contain valuable information about the composition and characteristics of food products (Wang *et al*., 2017).
Spectral pre-processing techniques play a crucial role in optimizing the quality of NIR modelling for food authentication. Pre-processing methods such as baseline correction, scatter correction, normalization, and outlier removal help to eliminate noise, enhance spectral features, and reduce unwanted variations. The appropriate

selection and combination of pre-processing methods significantly impact the accuracy and robustness of the subsequent modelling process (Rinnan *et al*., 2009). This needs a trial-and-error approach to pick the best pre-processing method(s) suitable for the given food matrix so as not to have under or over pre-processing of the spectra.

Given the large size of the NIR spectra generated (6000 data points for each NIR spectrum in this study), the general chemometric approach relies on data reduction using unsupervised methods such as PCA. The multicollinear nature of NIR spectra also benefits from PCA based data reduction. The principal components (PCs) thus obtained are used for supervised analysis. LDA, quadratic discriminant analysis (QDA), partial least squares regression-discriminant analysis (PLS-DA), soft independent modelling of class analogies (SIMCA), Orthogonal PLS-DA (OPLS-DA) are the most commonly used chemometric supervised techniques (Berrueta *et al*., 2007).

While traditional chemometric techniques have been widely used in NIR spectroscopy, machine learning algorithms have demonstrated superiority in food authentication tasks. Machine learning models, particularly those based on Support Vector Machines (SVM), possess remarkable capabilities in handling complex and high-dimensional NIR data used in this study. They can effectively identify patterns and classify samples based on their spectral fingerprints, providing higher accuracy and more robust predictions compared to traditional methods. Machine learning algorithms can learn from large datasets and adapt to various spectral variations, making them more versatile in handling different food authentication scenarios. Additionally, machine learning models can allow for an easy and quick integration of multiple spectral and non-spectral parameters, enabling a comprehensive analysis of food samples and enhancing the overall authentication process (Song *et al*., 2020; Mishra *et al*., 2022). So far, only one study has been published on application of ML on fish authentication, with no comparison with traditional chemometric techniques.

3. Sampling Plan

The selection of the sampling area and the number of samples is a crucial aspect of any study focusing on food traceability, particularly in the context of fish. It is essential to carefully consider these factors to ensure the accuracy and representativeness of the study results. When choosing samples, it is important to strive for an ideal representation of the natural variance of the analyte of interest within the target population. A representative sample can be defined as an aliquot of a material taken from a consignment, possessing all the essential characteristics of the bulk (Murray and Cowe, 2004).

In the case of fish, this natural variation can stem from factors such as fish age or size, the season of fishing, geographical location, fishing type (wild vs. farmed), freshness, food processing methods, water salinity, and water temperature, among others. Furthermore, the process of sampling can be influenced by various practical considerations. These include the available budget and timeframe for the project, the specific objectives of the study, and the logistical aspects associated with sample collection. Access to authentic samples, which accurately reflect the target population, also needs to be considered. By ensuring that the samples are selected in a manner that encompasses the key characteristics of the bulk, the study can provide reliable and meaningful insights into food traceability.

The samples from this study were obtained from local market in Portici (NA), Italy. The details of procurement of fresh anchovies from Tyrrhenian Sea and Adriatic Sea are provided in table 1.

Table 1: *Sampling plan of anchovies*

Month (2022)	Tyrrhenian Sea	Adriatic Sea
January	Trial 12 (n= 6)	-
February	Trial 14 (n= 12)	Trial 13 (n= 12), Trial 15 (n= 8)
March	Trial 16 (n= 11)	Trial 18 (n= 10), Trial 19 (n= 9)
April	Trial 20 (n= 9)	Trial 21 (n= 11)
May	Trial 23 (n= 9)	-
September	Trial 27 (n= 8)	Trial 26 (n= 9)
October	Trial 28 (n= 6)	Trial 29 (n= 9)
	N= 61	N= 68

Care was taken to keep the number of samples equal in anchovies from both locations to maintain sample balance. The final sampling, however, depended on the seasonal availability of fresh anchovies from either locations in the local market.

4. Materials and Methods

Fresh anchovies from Tyrrhenian Sea and Adriatic Sea were cleaned (removal of head, guts, bone, and internal viscera), washed with distilled water and sorted into different groups based on weight classes with a weight difference of 2g each.10-12 fishes from each group were homogenized together. The homogenate was freeze-dried and pulverized with ball-milling machine until 60% of it can pass through 100-mesh sieve and 80% can pass

through 60-mesh size sieve. Perkin Elmer FT-IR 9700 with NIRA (Near-Infrared Accessory) was used for spectra acquisition. Three spectra were acquired from each sample resulting in a total of 387 spectra (interleaved) from 129 samples. They were acquired over a wavelength of 1000-2500 nm with the resolution of 4 cm^{-1}, and 64 scans per spectrum.
The spectral pre-processing was performed on Unscrambler X (Version 10.4, 64 bit). Further data analysis was performed using Python 3.10.4. The Python libraries used for data analysis were Sci-Kit learn 1.2.2, Panda 2.0.2, Numpy 1.21.6, OS 2.1.4, and MatPlotLib 3.7.1. NIR spectra were pre-processed using techniques outlined in Table 2. Pre-processing techniques were chosen after a thorough review of literature for NIR based authentication of fish, meat, and other food products.

Table 2: *List of pre-processing techniques used for NIR spectra*

Pre-processing	Code
Raw spectra	Raw
Standard Normal Variate	SNV
Savitzky-Golay 1st derivate with 15 points of smoothening	SG-1d
Savitzky-Golay 2nd derivate with 15 points of smoothening	SG-2d
Multiplicative Scatter Correction	MSC
Standard Normal Variate + Savitzky-Golay 1st derivate with 15 points of smoothening	SNV+SG-1d
Standard Normal Variate + Savitzky-Golay 2nd derivate with 15 points of smoothening	SNV+SG-2d
Standard Normal Variate + Multiplicative Scatter Correction	SNV+MSC
Multiplicative Scatter Correction + Savitzky-Golay 1st derivate with 15 points of smoothening	MSC+SG-1d
Multiplicative Scatter Correction + Savitzky-Golay 2nd derivate with 15 points of smoothening	MSC+SG-2d

For chemometric modelling, the bulk of the NIR data with 6000 data points was reduced to 7 PCs by PCA. Initial PCA was checked for outliers and the whole process repeated to get final PCs for LDA. LDA was used to build a discrimination model using with training set and testing set of 0.8 and 0.2 respectively. For machine learning, SVM algorithm with liner and polynomial kernel using training and testing set of 0.8 and 0.2 respectively. Complete NIR spectra were used for SVM classification.

5. Results and Discussion

5.1 Spectral changes due to pre-processing

Raw NIR spectra, comprising numerous variables representing chemical constituents, suffer from multi-collinearity due to overlapping bands. Individual analytes may absorb at multiple wavelengths, leading to potential misreading. Weak absorption bands and non-linear scatter effects further complicate data interpretation. Baseline shifts and scatter effects, caused by light scattering and path length differences, introduce undesired variations (Rinnan *et al.*, 2009). Therefore, pre-processing is essential to mitigate these effects and enhance subsequent exploratory analysis. Image 1 shows the changes in the NIR spectra after spectral pre-processing.

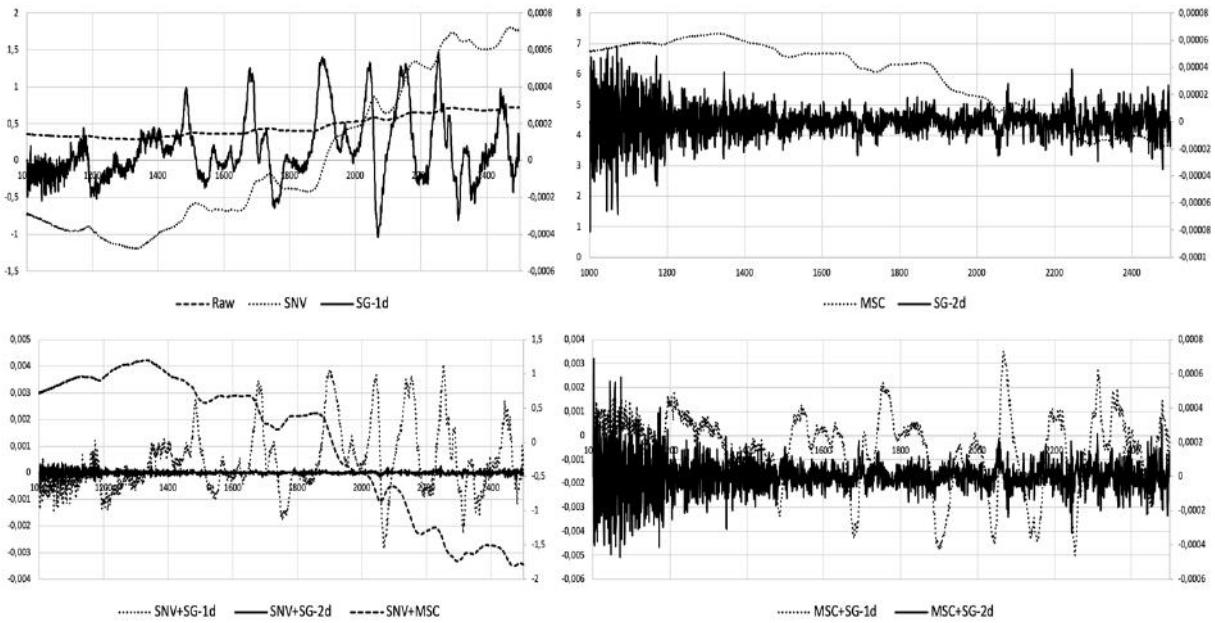

Figure 1: *Changes in NIR spectra after spectral pre-processing*

5.2 PCA-LDA analysis

Training score, testing score and cross-validation scores of PCA-LDA to discriminate between anchovies from Tyrrhenian Sea and Adriatic Sea are shown in Table 3.

All the pre-processed spectra performed better than raw spectra in general. The best performing pre-processing was that of SNV+SG-1d followed closely by MSC+SG-1d. As can be seen by Figure 1, SG-1d and SG-2d led to a large-scale spectral transformation. However, 2nd derivate spectra appeared over-pre-processed and thus did not perform as well as 1st derivate. The multicollinearity of NIR spectra in these cases of PCA was only reduced, not completely eliminated and thus the discrimination was not entirely accurate. Reduction in accuracy can also be attributed to seasonal and size differences between the anchovies from same location. Anchovies have been documented to have seasonal changes based on changes in food availability and size. Since, the sampling was completely random and did not pick anchovies of one size, the variance in NIR data from the same batch of anchovies was high.

Table 3: *Results of PCA-LDA discrimination on raw and pre-processed NIR spectra*

Pre-processing	Training Score	Testing Score	Cross Validation
Raw	0.74	0.67	0.65
SNV	0.87	0.90	0.91
SG-1d	0.89	0.90	0.92
SG-2d	0.81	0.85	0.83
MSC	0.84	0.89	0.87
SNV + SG-1d	0.93	0.96	0.95
SNV + SG-2d	0.82	0.86	0.84
SNV + MSC	0.89	0.92	0.92
MSC + SG-1d	0.94	0.95	0.92
MSC + SG-2d	0.80	0.85	0.81

5.3 Machine learning analysis

SVM was chosen over all other ML approaches in its efficiency in handling large sets of multicollinear nature, like the NIR spectra. Linear and polynomial kernels were used for classification between Tyrrhenian and Adriatic anchovies. The classification results are presented in Table 4.

Table 4: *Results of SVM classification on raw and pre-processed NIR spectra*

Pre-processing	SVM linear kernel		SVM polynomial kernel	
	Train	Test	Train	Test
Raw	0.64	0.54	0.69	0.55
SNV	0.82	0.91	0.89	0.90
SG-1d	0.89	0.96	0.98	1.00
SG-2d	0.80	0.76	0.81	0.85
MSC	0.81	0.80	0.89	0.78
SNV + SG-1d	1.00	0.97	1.00	1.00
SNV + SG-2d	0.86	0.93	0.88	0.89
SNV + MSC	0.85	0.80	0.89	0.81
MSC + SG-1d	0.98	1.00	1.00	0.99
MSC + SG-2d	0.93	0.91	0.92	0.82

In general, polynomial kernel performed better than linear kernel in this case since the data was highly multicollinear and the whole spectra was used instead of PCs. The pre-processing techniques remarkably well, both individually and in combination. Most pre-processing techniques had accuracy of more than 0.90 in training and testing set. SNV+SG-1d performed the best followed by MSC+SG-1d and SG-1d.

5.4 Comparison between PCA-LDA, SVM linear and SVM polynomial

SVM polynomial outperformed LDA-PCA and SVM linear in terms of accuracy of classification of anchovies between the two locations. SVM was better able to capture and process the non-linear seasonal and size differences between anchovies from the same location thereby picking up intricate relations in the dataset. Use of SVM also removed the step of data reduction and made the whole step of data analysis shorter and less time consuming. This was possible to high computing ability of ML techniques. LDA relied on certain assumptions about the data distribution and relationship between variables given by PCA. SVM models, on the other hand, are more flexible and can make predictions without strong assumptions, reducing the risk of model bias (Song *et al*., 2020; Mishra *et al*., 2022). Some pre-processing techniques performed better with PCA-LDA than SVM, which can be attributed

to the reduction in multicollinearity by PCA.
Between the two SVM kernels, polynomial kernel outperformed the linear kernel, since most relationships in NIR spectra are non-linear which were better captured by the polynomial kernel. The polynomial kernel implicitly maps the original feature space into a higher-dimensional feature space. By doing so, it enables SVM models to find non-linear decision boundaries that can effectively separate data points in the transformed space. This ability to operate in a higher-dimensional feature space can help SVM models better handle multicollinear NIR datasets, where the variables may not be linearly separable in the original feature space (Patle and Chouhan, 2013). In addition, the polynomial kernel allows for the detection and representation of interactions between variables. In the case of multicollinearity, where variables may be correlated with each other, the polynomial kernel can capture the complex interactions and dependencies between these variables. This flexibility enables the SVM model to better understand the underlying structure of the data and make more accurate predictions (Ben-Hur *et al*., 2008).
The anchovies from two locations in Mediterranean Sea were thus classified due to changes in their chemical composition which can be further attributed to differences in food availability between the two locations. This was shown by analysis of nutritional and fatty acids composition of fish. Large scale differences were observed in fatty acid composition, especially omega-3 fatty acids which were then measured by the NIR spectra. (The nutritional data was not complete on the day of submission and thus not shown here).

6. Conclusions and Future Perspectives

Spectral pre-processing in as integral part of NIR based food authentication and depends on the food matrix and instrument used for NIR spectra. SNV+SG-1d, followed closely by MSC-SG-1d and SG-1d turned out to be the best spectral pre-treatment based on discrimination and classification results by PCA-LDA and SVM respectively. The SVM polynomial kernel outperformed LDA-PCA and SVM linear in accurately classifying anchovies between different locations. SVM exhibited superior capability in capturing and processing the non-linear seasonal and size differences within anchovies from the same location, uncovering intricate relations in the dataset and eliminated the need for data reduction. Overall, the polynomial kernel outperformed the linear kernel by effectively handling the non-linear relationships present in NIR spectra by mapping the feature space into a higher-dimensional space enabling the SVM models to discover non-linear decision boundaries and better handle multicollinear NIR datasets.
Although ML handled the NIR data considerably well, there is still scope of bettering the process by spectral selection to further reduce the bulk of data and ease the process of data fusion. Data fusion, which involves using data from two more analytical techniques, can create more robust and chemically comprehensive model.

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Vegetable agri-food by-products: a source of functional ingredients for the production of high added value foods

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This PhD thesis aimed to formulate high added value foods through the exploitation of plant-based waste and by-products to be used as innovative ingredients or a source from which to extract bioactive molecules in a sustainable way. Specifically, one of the main research activities was focused on the valorisation of globe artichoke roots as an alternative and sustainable source of inulin by green extraction and its application as ingredient for the production of functional fresh pasta.

Scarti e sottoprodotti vegetali come fonte di ingredienti funzionali per la produzione di prodotti alimentari ad alto valore aggiunto

Il presente progetto di dottorato ha avuto lo scopo di formulare alimenti ad alto valore aggiunto valorizzando scarti e sottoprodotti di origine vegetale mediante il loro utilizzo come ingredienti funzionali o fonte da cui estrarre molecole bioattive sfruttando metodiche green. Nello specifico, una delle principali attività di ricerca ha riguardato la valorizzazione di radici di carciofo come fonte alternativa e sostenibile per l'estrazione di inulina e sua applicazione come ingrediente innovativo per la produzione di pasta fresca funzionale.

Key words: plant-based; waste; by-products; green extraction; inulin; pasta.

1. Introduction

In recent years, the increasing awareness of consumer about the relationship between diet and health has led to the development of new foodstuffs. Vegetable and fruit processing industries generate a large amount of waste and by-products, including peels, roots, seeds, husks and pomace, representing a source of macromolecules and bioactive compounds like dietary fibre, proteins, essential fatty acids, polyphenols and other phytochemicals. All these molecules are well-known to have health beneficial effects, such as regulation of metabolic process, antioxidant and anti-inflammatory activity (Gómez *et al.*, 2018). Italy is one the major producer of globe artichoke, which roots represents an important agricultural waste. Globe artichoke roots are rich in inulin, a fibre characterized by a mixture of oligo and polysaccharides consisting of a variable number of *d*-fructose units. The degree of polymerisation of this fibre affects both technological and nutritional properties; generally short-chain inulin is used as an alternative low-calorie sweetener, while long-chain inulin mixed with water form a gel network which can be used as a fat replacer or texture modifier. Moreover, the peculiar bond configuration confers to inulin a prebiotic characteristics (Raccuia *et al.,* 2010; López-Molina *et al.,* 2005). A regular intake of prebiotics has several benefits like modulation of hyperglycaemia, reduction of LDL cholesterol and serum lipids, enhancement of immune system. Fresh pasta is a product spread worldwide and daily consumed, rich in carbohydrate but relatively poor in other nutrients (dietary fibre), representing a suitable food to deliver functional ingredients like inulin (Bianchi *et al*., 2022). Several studies have tried to improve the nutritional profile of pasta by adding soluble and insoluble fibre, finding conflicting results in terms of technological quality.
In this perspective, this oral communication reports the main results of the following activities:
A1) green extraction of inulin from globe artichoke roots;
A2) characterisation of extracted inulin;
A3) fresh pasta production and evaluation of the inulin addition on textural, sensory and nutritional properties.

2. Materials and Methods

2.1 Extraction and characterisation of inulin from globe artichoke roots

Inulin green extraction was carried out according to Difonzo *et al.* (2022). For the extraction process it was used water (pH = 6.8) as solvent with a ratio solid to water of 1:16 (*w/v*). The extraction consisted of 2 h of brewing at 80 ºC, followed by filtration and precipitation steps. Afterwards, the sample was centrifugated, washed with ethanol, centrifugated and the pellet dried overnight. Inulin yield (%) = (weight of inulin (g))/(weight of artichoke roots (g)) × 100. Moisture content was determined with a moisture analyser and water activity (a_w)

using a hygrometer. Identification and quantification of inulin was carried out through high-performance liquid chromatography (HPLC) equipped with a refractive index detector (RID) and a cationic exchange column. The analysis was conducted isocratically using Milli-Q water as mobile phase with a flow of 0.6 mL min^{-1}, column temperature 80 ºC and RID 35 ºC, commercial inulin with high degree of polymerisation and high purity was used as a standard. Number average degree of polymerisation (DPn) and weight average degree of polymerisation (DPw) of extracted inulin were evaluated using a gel permeation chromatography. The injection volume was 100 µL and flow rate of 0.8 mL DPn and DPw were calculated using the following equation: Mn = 180 + 162 × (DPn – 1), Mw = 180 + 162 × (DPw – 1); Mn is the number average molecular weight, while Mw is the weight average molecular weight.

2.2 Fresh pasta preparation

Inulin-enriched fresh pasta was produced by replacing 5% (P5), 10% (P10) and 15% (P15) of durum wheat semolina with inulin. Control pasta (PC) was produced with 100% of durum wheat semolina. The flour was mixed with an adequate amount of water and manually kneading, the dough was then laminated and cut to produce tagliatelle pasta. Tagliatelle were left dried until reaching 26-28% of moisture content and a_w values ranging from 0.92-0.97, according to the Italian legal requirements for fresh pasta production and marketing.

2.3 Fresh pasta characterisation

2.3.1 Cooking properties of fresh pasta

Pasta samples were cooked in boiling distilled water at 1:10 (*w/v*), without the addition of salt, according to Pasqualone *et al.* (2016). The optimum cooking time (OCT) was determined according to the AACC 16-50 official method (AACC, 2000), cooking loss (CL), water absorption (WAI) and swelling index (SI) was determined according to Bustos *et al.* (2011). Inulin loss (IL) in cooking water (g 100 g^{-1} of pasta) was determined analysing its inulin content by a cationic exchange HPLC following the same methods described in section 2.1.

2.3.2 Instrumental and sensory analysis of fresh pasta

Colour (L*, luminosity; a*, redness; b*, yellowness) of raw and cooked fresh pasta was evaluated using a colorimeter. Pasta firmness, cooked at their OCT, was assessed measuring the maximum force (N) required to cut 5 strands of pasta at a speed of 0.17 mm s^{-1}. The microstructure and the surface characteristics of raw pasta were studied with a Zeiss Sigma 300 VP field-emission gun scanning electron microscope (FEG-SEM) equipped with a secondary electrons detector (SE).

Sensory analysis was conducted on fresh pasta, cooked at OCT, by a panel of eight trained testers which evaluated the colour, odour, taste, bulkiness, adhesiveness and firmness, using a structured scale ranging from 1 to 10.

2.4. Proximate composition and functional properties

Proteins (total nitrogen × 5.7), ashes, lipids and total dietary fibre content were determined using the AOAC method 979.09, 923.03, 945.38F, and 991.43, respectively (AOAC, 2006). Moisture content was determined by a moisture analyser, while carbohydrate content was determined as difference. *In vitro* starch hydrolysis was determined according to Liljeberg et al. (1996). Free glucose was determined using an enzyme-based kit and converted into hydrolysed starch in pasta. Control white bread was used as a control to estimate the hydrolysis index (HI) = 100. The predicted glycaemic index (pGI) was calculated using the equation pGI = 0.549 × HI + 39.71 (Capriles *et al.,* 2013).

To evaluate the prebiotic activity of inulin-enriched fresh pasta, samples were subjected to *in vitro* gastrointestinal digestion according to the method used by Kamiloglu and Capanoglu (2014). Twenty-two probiotic strains and one strain of *Escherichia coli* (*E. coli*) were used to carry out the experimental in faecal medium (FM). The FM was constituted as described by Vacca *et al*. (2021) in absence of carbohydrates, FMPC (FM + control pasta), FMP5 (FM + pasta with 5% of inulin), (FM + pasta with 10% of inulin) and FMP15 (FM + pasta with 15% of inulin). Prebiotics and *E. coli* were incubated in FM at density of 7 UCF mL^{-1}. After the incubation, plate counts for lactic acid bacteria and *E. coli* were respectively made in De Man, Rogosa, and Sharpe agar (MRS) and Violet Red Bile Glucose agar (VRBGA). Probiotic growth was also profiled in terms of ΔpH, as the difference between final (36 h) and initial (pH 7.0 ± 0.02) values of pH.

2.5 Statistical analysis

The experimental data were subjected to one-way and two-way ANOVA, followed by a Tukey's HSD test. The two-way ANOVA analysis was carried out considering the rate of substitution and the physical state of pasta (raw and cooked) as factors. Significant differences among the values of all the parameters were determined at *p*-value < 0.05 by the Minitab 17 Statistical Software (Minitab, Inc., State College, PA, USA, 2010).

3. Results and Discussion

3.1 Inulin powder characterisation

The extraction yield of inulin can be influenced by several factors such as: temperature, time of extraction or the ratio solid/liquid (Rubel *et al.,* 2018). From the data collected, the extraction yield of inulin from globe artichoke roots was 23.37 g 100 g^{-1}, with a purity level of 89%, estimated in comparison to commercial inulin used as a standard. Moreover, the extracted inulin showed a DPn and DPw equal to 45 and 60, respectively, a moisture content of 6% and a_w of 0.40, values which assure high glass transition temperature, lower cohesiveness and good physical and microbiological stability (Jirayucharoensak *et al.,* 2019).

3.2 Quality characteristics of fresh pasta

Pasta cooking properties are of great importance for ensuring consumers acceptability. Table 1 shows the OCT, SI, WAI and CL of fresh pasta samples. OCT was set for each pasta sample, observing a slight increase in P10 and P15; WAI, showed no significant differences among the sample, while a lower values of SI were found for P10 and P15 than PC. As regard CL, good quality pasta should not have a CL higher than 7-8%, our results showed a CL between 2.37% for PC and 3.62% for P15. Specifically, IL accounted for 1.01 g 100 g^{-1} for P5, 1.45 g 100 g^{-1} for P10 and 2.19 g 100 g^{-1} for P15. The low SI and CL values obtained can be related to the encapsulation of starch granules into fibre reticule, which limited the penetration of water into starch granules and provided an extra support to the protein network, reducing the solid loss (Liu *et al.,* 2016). However, discordant results were found in literature probably due to the different production process, the fibre added and their interaction with other ingredients (Simonato *et al.,* 2019; Zarrough *et al.,* 2022).

Table 1 *Cooking properties of fresh pasta.*

Sample	OCT (min)	WAI (g 100 g^{-1})	SI	CL (g 100 g^{-1})	IL (g 100 g^{-1})
PC	6.30	73.20 ± 0.01 a	1.56 ± 0.03 a	2.37 ± 0.05 d	-
P5	6.30	73.24 ± 0.10 a	1.53 ± 0.04 ab	2.70 ± 0.14 c	1.01 ± 0.03 c
P10	6.45	72.51 ± 0.62 a	1.48 ± 0.03 b	3.11 ± 0.07 b	1.45 ± 0.07 b
P15	6.45	73.24 ± 0.20 a	1.47 ± 0.04 b	3.62 ± 0.06 a	2.19 ± 0.12 a

PC, control pasta without inulin addition; P5, pasta with 5% of inulin added; P10, pasta with 10% of inulin added; P15, pasta with 15% of inulin added. OCT, optimal cooking time; WAI, water absorption index, SI, swelling index; CL, cooking losses; IL, inulin losses. Values are expressed as mean ± standard deviation; different letters in the same column mean significant statistical differences ($p < 0.05$) to one-way ANOVA followed by Tukey's HSD test.

Table 2 shows the colour and firmness of raw and cooked pasta. According to two-way ANOVA, both the cooking process and inulin addition significantly affect the colorimetric parameters. Both in raw and cooked pasta occurred a reduction of L* and b* with higher substitution rate of durum wheat semolina with inulin. The red index (a*) followed the same trend for cooked pasta, while an opposite trend was observed considering the increasing rate of substitution. Firmness of pasta is strictly related to protein matrix development during pasta production and hydration level of starch granules (Simonato *et al*., 2019). Cooked P10 and P15 showed higher firmness than PC and P5, this increase can be attributed to the lower values of SI found and to a rearrangement of pasta structure as observed through SEM micrographs of raw pasta (Figure 1), where P10 and P15 exhibited the presence of a reticulated structure, supporting the hypothesis discussed above.

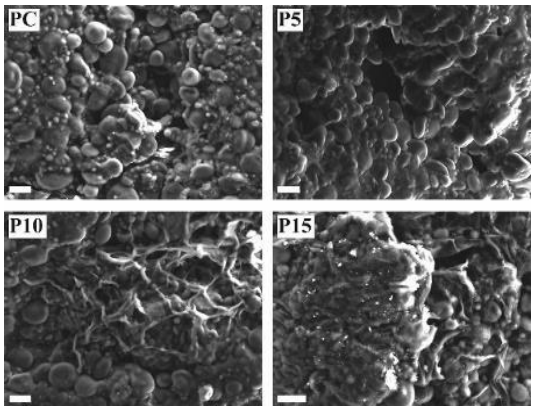

Figure 1 *SEM micrographs of raw pasta of Control pasta (PC), pasta with 5% of inulin (P5), 10% (P10) and 15 % (P15).*

Table 2 *Colour and textural properties.*

		L*	a*	b*	Firmness
Raw pasta	PC	79.65 ± 0.09 a	2.21 ± 0.07 e	33.57 ± 0.46 a	18.25 ± 0.45 b
	P5	74.57 ± 0.19 c	3.21 ± 0.02 c	30.49 ± 0.26 b	19.63 ± 0.50 a
	P10	72.02 ± 0.38 e	3.89 ± 0.10 b	28.52 ± 0.19 c	19.89 ± 0.05 a
	P15	70.72 ± 0.25 f	4.48 ± 0.08 a	27.62 ± 0.09 cd	19.77 ± 0.23 a

Cooked pasta	PC	78.89 ± 0.43 b	0.30 ± 0.02 f	27.11 ± 0.56 d	5.85 ± 0.36 d
	P5	73.69 ± 0.18 d	2.00 ± 0.11 e	24.13 ± 0.41 e	6.22 ± 0.26 d
	P10	72.24 ± 0.06 e	2.82 ± 0.11 d	24.18 ± 0.33 e	7.37 ± 0.48 c
	P15	68.12 ± 012 g	3.79 ± 0.10 b	22.29 ± 0.43 f	7.25 ± 0.37 c
p-value	P *C	<0.0001	<0.0001	<0.0001	<0.05

PC, control pasta without inulin addition; P5, pasta with 5% inulin added; P10, pasta with 10% inulin added; P15, pasta with 15% inulin added. P, percentage of inulin addition; C, cooking process. Values are expressed as mean ± standard deviation; different letters in the same column mean significant statistical differences ($p < 0.05$) according to two-way ANOVA.

The sensory analysis results are reported in Figure 2. The increasing rate of substitution of durum wheat semolina with inulin did not cause significant changes in sensory perceptions. Colours and texture scores were consistent with instrumental evaluation. Significant differences were found for taste of all inulin-enriched samples than control, while panellists perceived an odour significant different only for P15.

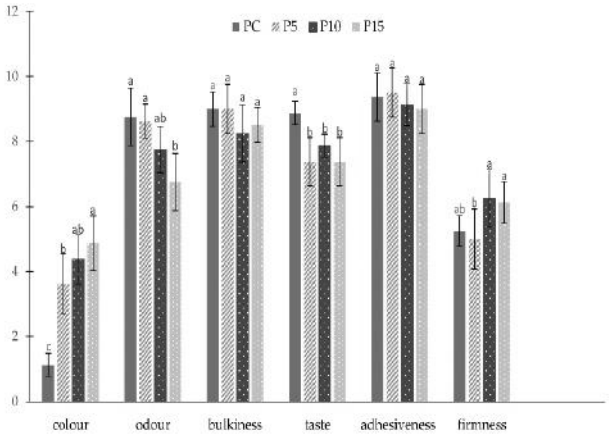

Figure 2. *Sensory analysis of fresh pasta.*

3.3 Functional properties of fresh pasta

The proximate composition of pasta showed a decline in protein content of inulin-enriched pasta (~ 9 g 100 g^{-1}) than PC (~ 11 g 100 g^{-1}), due to a rise in total dietary fibre content, which reached values of 3.44 g 100 g^{-1} in P5, 8.16 g 100 g^{-1} in P10, and 12.41 g 100 g^{-1} in P15. Therefore, the results in terms of total dietary fibre allow to label P5 as a "source of fibre", while P10 and P15 as pasta having "high fibre content" according to Reg. (EU) 1924/2006, enhancing the nutritional value of fresh pasta. Moreover, higher ash and lower lipid content were found in P10 and P15 than P5 and PC (data not showed).

Cooked pasta was analysed for *in vitro* starch hydrolysis, as shown in Figure 3, the addition of inulin in fresh pasta promoted the decrease in HI and pGI. The HI and pGI values of fresh pasta samples enriched with inulin (P5, P10 and P15) were significantly lower than PC. P5 reached a significantly lower value of pGI compared to the control (PC): 63.01 and 66.54%, respectively. A higher concentration of inulin resulted in a further decrease of HI and pGI, as shown in P10 and P15. In fact, the gelling effect of these fibres causes the formation of a film on the walls of the stomach and gut with consequent lower absorption of fats and sugars (Kumar *et al.,* 2012).

The prebiotic activity of inulin-enriched pasta was assessed *in vitro* in terms of probiotics growth and the inhibition of *E.coli* when co-cultured with probiotics (Figure 4). The addition of pasta in the batches (FMP) was sufficient to increase (~0.5 log_{10} CFU mL^{-1}) the cell density of all the tested prebiotics, due to the presence of fructans and arabinoxylans naturally occurring in wheat. However, the presence of 3 g L^{-1} of inulin in FMP15 was able to increase of > 0.5 cycle the cell density of 50% of used probiotics and more than 1 cycle of 27 % of used strains. No significant difference were found comparing the cell density of *E.coli* in FMP to FMP5. However, more than 50% of used prebiotics were able to significantly decrease the cell density of *E.coli* in FMP15, and 36 % in FMP10. These results are in line with those stated by Kareem *et al.* (2014), who reported that the combination of probiotics with prebiotics *in vitro* exhibited a great inhibition of pathogens due to a synergic

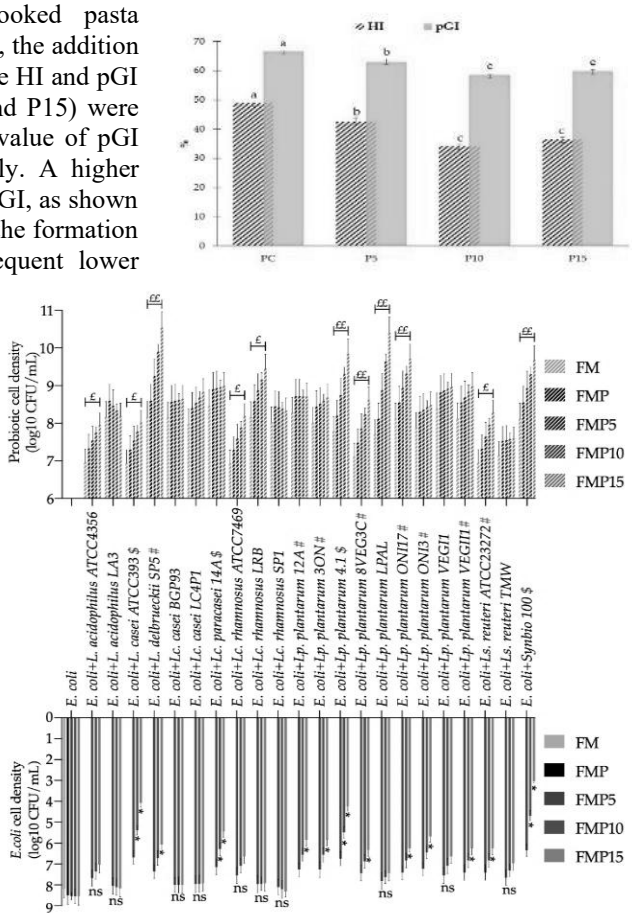

Figure 4. *Cell density of 22 probiotics co-cultured with E.coli in faecal batches not containing carbohydrate (FM), with the addition of pasta without inulin (FMP), pasta with 5% of inulin (FMP5), 10 % (FMP10) and 15 % (FMP15).*

effect.

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Probiotic bacilli incorporation in foods: is really so easy?

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The objective of this PhD thesis was to evaluate the resistance of Bacillus spp. strains with claimed probiotic properties added to different foods (pasta and croissants) at the beginning and the end of the products' shelf life and after exposure to simulated GIT conditions.

Alimenti arricchiti con bacilli probiotici: è davvero così facile?

L'obiettivo di questa tesi di dottorato è stato quello di valutare la resistenza di ceppi di *Bacillus* spp. con riconosciute proprietà probiotiche, aggiunti a diversi alimenti (pasta e croissant), durante la conservazione e dopo l'esposizione a condizioni di digestione simulata.

Key words: Probiotic bacilli; Pasta; Croissant; Spores' germination; Cold storage; Food matrix.

1. Introduction

In accordance with the PhD thesis project previously described, this oral communication reports the main outcomes obtained by the following three activities:
A1) assessment of the spores' survival along GIT when incorporated in different matrices;
A2) evaluation of the effect of different thermal treatment (baking and boiling);
A3) monitoring of the storage at -18°.

2. Probiotic Food development

The development of probiotic foods, especially those with acidic pH, low water activity, or that will be exposed to thermal processes with temperatures exceeding 60°C, is a great challenge (Marcial-Coba et al., 2019). Spore-forming bacteria with claimed probiotic properties can help to overcome various technical troubles thus expanding the possibility of applying probiotic microorganisms to a broader range of products, including acidic beverages, thermally processed dairy products, and bakery goods.

3. Spore and Germination

From a probiotic perspective, spores and vegetative cells have separate but complementary functions (Bernardeau et al., 2017). Both spores and vegetative cells can promote some characteristics, such as immunomodulation and adsorption, whilst others, such as antimicrobial activity, are a distinctive trait of vegetative cells (Bernardeau et al., 2017). The germination process that ensures metabolic activity is highly dependent on the strain itself, GIT location, environmental conditions, as well as the conditions applied during the sporulation process prior to ingestion (Bernardeau et al., 2017). In general, there is clear evidence that *Bacillus* spores can germinate in GIT conditions, nonetheless, this fact should be confirmed during the food development process and before any potential *in vivo* test. Based on the previous considerations, the development of foods fortified with spores ought to ensure the germination of the spores along the gastrointestinal tract.

4. Experimental Procedure

Pasta and croissants were used as food matrices for the spore addition. Pasta, in detail "spaghetti" and "sedanini rigati", was prepared by means of a professional electric pasta machine (Dolly – La Monferrina, Padova, Italy) by using tap water (in which spores were resuspended) and commercial semolina. Croissants, were prepared in the laboratory and subsequently at a nearby patisserie (De Girolamo, Marigliano, Naples, Italy). In the last test, croissants were filled with probiotic chocolate cream, immediately placed in a blast chiller, and kept at -18°C for analysis. At each sampling point, pasta and croissants were subject to simulated digestion immediately after cooking. The protocol was that described by Ricciardi et al. (2014) with some modifications.
Furthermore, spores of the three probiotic bacilli were mixed with different matrices: starch, butter, and gluten. Gluten and starch were combined with sterile water (40%) to create a dough. Initial microbial loads were fixed at 10^5 CFU g^{-1}. Food matrices were then monitored during freezing.

5. Materials and Methods

Poliantibiotic *Bacillus* (*B.*) clausii strain was used as spores' suspension as available in Enterogermina and is

herein named *B. clausii* SA (Sanofi Aventis). *B. clausii* UBBC07 and *B. coagulans* Unique IS2 were provided as dried powders containing 1.5 x 10^{10} spores by Unique Biotech. In all cases, the spores' content was checked by plate counting onto the LB (Lennox) medium (Tryptone 10 g L^{-1}, Yeast extract 5 g L^{-1}, NaCl 5 g L^{-1}) as well as on the same medium after treatment at 75°C in a shaking water bath for 30 min at 50 rpm, followed by immediate cooling below 45°C (LB75°C). Vegetative cells deriving by spores' germination along GIT or during the process were separately counted on the Spizizen medium (SM) with glutamic acid (1%) as nitrogen source (Anagnostopoulos & Spizizen, 1961). Significant differences among data were tested by ANOVA (Tukey). The significance level was $p \leq 0.05$ throughout the analyses. Data elaboration was carried out using XLStat (version 2012.6.02), an add-in software package for Microsoft Excel (Addinsoft Corp., Paris, France).

5. Results and Discussion

5.1 Spores' survival along GIT and storage when incorporated in pasta and croissants

The three bacilli cultures' spores were added to two types of pasta and to a chocolate cream that was used to stuff croissants (Fig. 1). All spores that were added to pasta were induced to germinate, and vegetative cells were able to grow on minimum media, demonstrating that the induction to germination most likely occurs during the boiling process used to cook pasta. The population levels of the three strains decreased in pasta with counts following GIT, being at least one Log lower (Fig. 1). However, up to 60 days, counts on the two media before or after GIT were stable ($p < 0.05$). When added to the chocolate cream used to fill croissants, the spores of the three bacilli performed entirely differently. Results at time 0, with the exception of strain B. *clausii* UBBC 07, make it possible to infer that no spores germinate during GIT. The three types of probiotic products were subject to simulated digestion after 30 and 60 days of storage at room temperature and at -18°C for pasta and croissants, respectively. In pasta, all spores germinated independently by the strain and the pasta shape. Pasta boiling is able to induce complete spores' germination, and the vegetative cells' survival along GIT is consistent with data reported by Konuray and Erginkayafor (2020) for *B. coagulans* GBI-30 in spaghetti. The main differences in pasta appear to be strain-dependent: strain's UBBC 07 tolerance to GIT seems to be reduced by storage. On the other hand, *B. coagulans* Unique is the sole strain that experienced a drop in SM counts after 30 days of storage, but only when added to Sedanini rigati, probably as a result of the longer cooking time. This shows that there are multiple factors that affect how spores react to thermal treatments. The combination of chocolate filling and oven heating was found to be a better matrix. Probiotic bacilli displayed optimal tolerance to GIT conditions: with the exception of strain *B. coagulans* unique IS2, counts on LB and on the same medium after the thermal treatment produced overlapping population levels after GIT at 30 and 60 days. Conversely, minimal medium counts turned out to be somewhat unpredictable. For both *B. clausii* strains, populations on SM dropped under the threshold of detection of the method after two months. In other words, the harsh environmental conditions encountered along GIT seemed to have prevented the spores' germination (Fig. 1). Only for *B. coagulans* Unique IS2 germination seems to be triggered by GIT at 30 and 60 days.

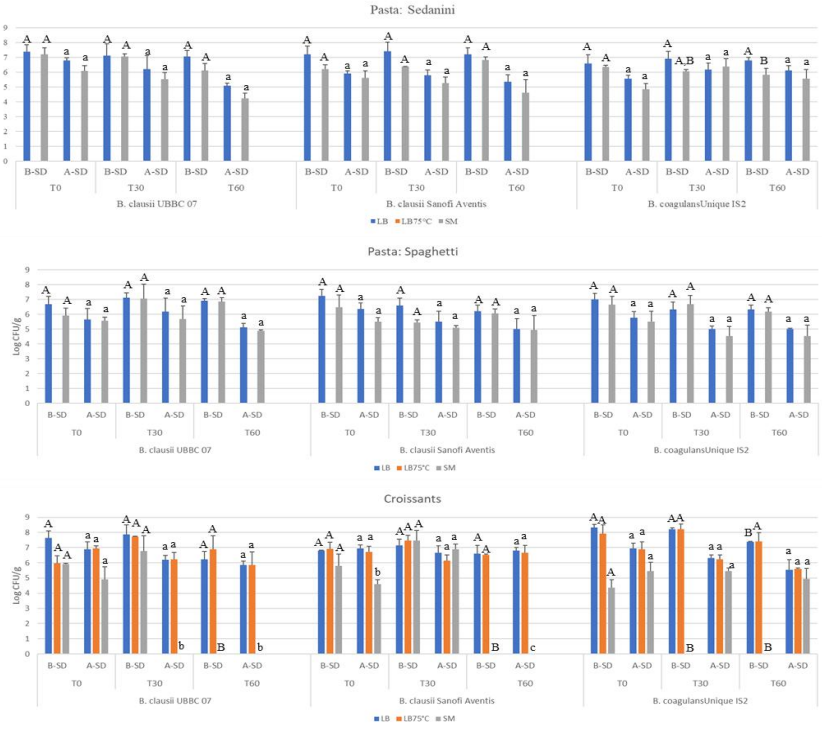

Figure 1 *Spores counts before (B-SD) and after simulated (A-SD) digestion of spores incorporated in pasta or in chocolate croissants' filling at time 0 and after 30 and 60 days of storage at room temperature (pasta) or -18°C (croissants). For data with the same letter, differences during storage are not statistically significant ($p < 0.05$).*

5.2 Spores' behaviour at -18°C

A second batch of croissants was made, and the product's storage for up to four months was monitored (Fig. 2). After baking, the LB and spore counts were stable until the end of monitoring ($p < 0.05$), showing that this kind of product may be profitably utilized to vehicle probiotic bacilli. The graph in Figure 2 shows dashed lines for counts after GIT transit, which are consistently lower than counts before GIT. The most surprising information was the spores' ability to germinate on minimal medium: in every case, after two months, spores appeared to lose this capacity. In both strains, *B. clausii* SA and *B. coagulans* Unique IS, the ability to germinate after three months is only triggered by GIT transit. *B. clausii* UBBC 07 exhibited the most peculiar behavior: counts on SM increased back at the end of monitoring (Fig. 2).

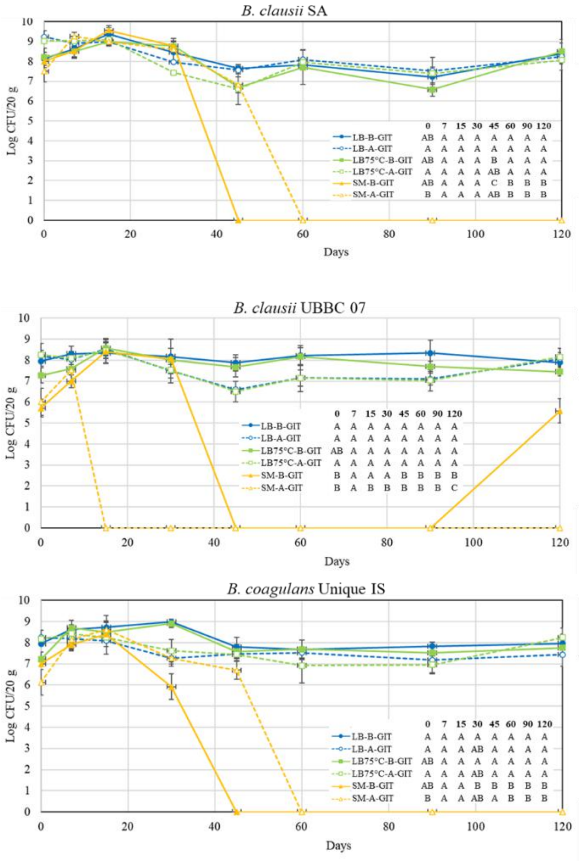

Figure 2 *Monitoring of spores in the chocolate filling of croissants for up to 120 days. Counts before (B-GIT) and after (A-GIT) simulated digestion are reported as full and dashed lines respectively. For data with the same letter, differences during storage are not statistically significant ($p < 0.05$).*

5.3 Influence of the thermal treatment

The completely different behaviour of spores incorporated in chocolate might be likely related to more than one factor, but basically, the lipidic nature of the matrix, storage at freezing temperatures, or the type of cooking.

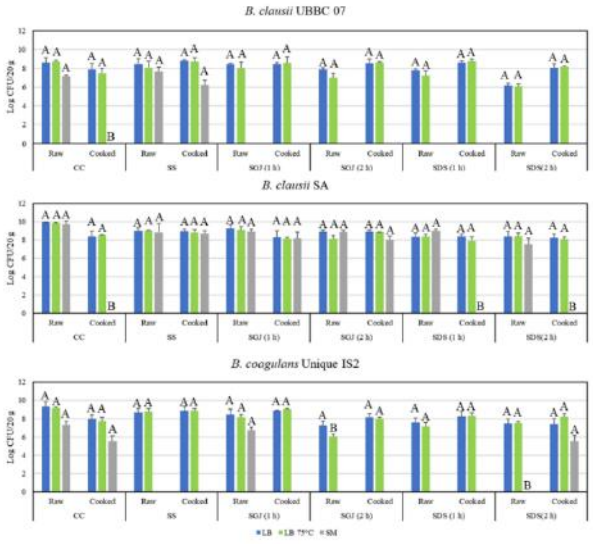

Figure 3 *Evaluation of probiotic spores during simulated digestion of raw and baked croissants' chocolate filling after 120 days at -18°C. For data with the same letter, the differences during GIT are not statistically significant ($p < 0.05$). Raw and Cooked croissants were separately subject to Anova.*

In order to exclude the influence of the baking, croissants after 120 days of storage at -18°C were subject to

simulated digestion before and after cooking (Fig. 3). For all strains, a number of spores higher than 80% were able to germinate on the SM in raw croissants. Upon cooking this percentage is significantly lower for *B. coagulans* IS2, while no germination occurs for both *B. clausii* strains. Stressed encountered during simulated digestion lowered this percentage and, after simulated duodenum bacilli counts on SM were always under the threshold of detection except that for *B. coagulans* IS2. The stress of cold/hot coupled with the hostile conditions prevailing along GIT may be the cause of this phenomenon. For *B. clausii* SA, the thermal treatment seems to be the sole cause for the loss of viability: when submitted as raw to simulate digestion spores kept the ability to germinate on minimal medium throughout the simulated digestion (Fig. 3).

5.4 Influence of the storage at -18°

At a starting concentration of approximately 9 Log CFU mL^{-1}, spores were resuspended in water and monitored for up to 180 days at -18°C (Fig. 4). During freezing, spores experienced a loss of viability, among all *B. clausii* UBBC 07 suffered significantly higher losses. After the simulated digestion, both Unique Biotech strains lost more, with counts falling below 80%. The monitoring of the counts on minimal medium produced the most peculiar data. After two weeks, the spores' capacity to germinate on SM diminished and they ceased to be countable. By the 90th day, it appears that the lengthy storage has encouraged germination since the proportion of spores that can grow on SM has gradually increased. Additionally, in these circumstances, the spore germination appears to be triggered by the GIT transit (Fig. 4). Spores were simultaneously added to three distinct matrices, including gluten, starch, and butter (Fig. 5). The idea that germination is influenced by the elements in the counting medium is supported by the lack of any noticeable differences between counts of spores on LB and those on LB75°C. The worst matrix is gluten, which after a month prevented germination on the minimal medium. But in this instance, the GIT appears to be what triggers the spores to germinate (Fig. 5).

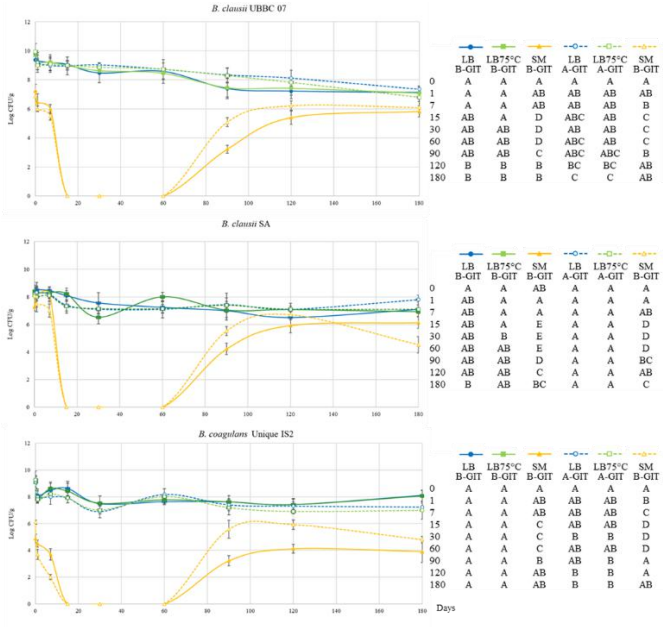

Figure 4 *Monitoring of spores resuspended in water and stored for up to 180 days at -18°C. Counts before (B-GIT) and after (A-GIT) simulated digestion are reported as full and dashed lines, respectively. For data with the same letter, differences during storage are not statistically significant ($p < 0.05$).*

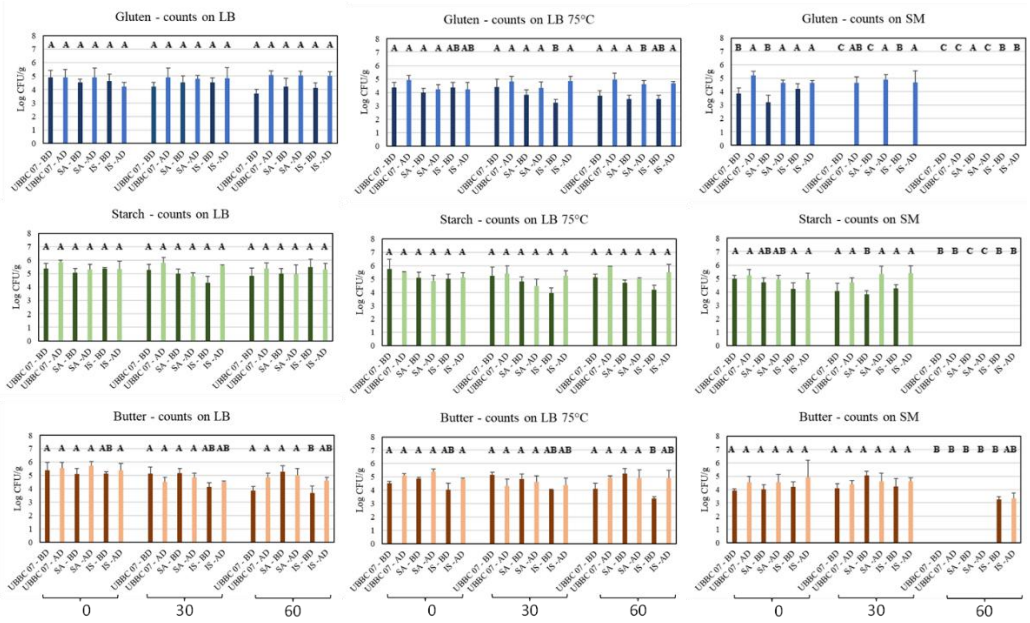

Figure 5 *Counts before (BD) and after (AD) simulated digestion of spores resuspended in gluten, starch, or butter and stored for up to 60 days at -18°C. For data with the same letter, differences during storage are not statistically significant ($p < 0.05$).*

6. Conclusions and Future Perspectives

Unquestionably important aspects that may affect the spores' germination and outgrowth, and consequently its maximum recovery, include the medium's components, pH, and whether it is nutritionally too rich or too poor. In order for bacilli to be beneficial, they must regain their metabolic function once they have entered the host. For this reason, it is crucial to know how many spores may germinate during food ingestion and digestion. Bacilli have been included in a vast array of diverse dietary matrices, but in every instance, spores have only been counted on complex media, putting information in jeopardy from germination triggered by nutrients in the counting medium. In this context, using minimal media may be a helpful strategy. In the current work, probiotic bacilli were added to a frozen ready-to-bake product for the first time. This technology is becoming more and more popular among many for several *viennoiseries* because of how convenient it is. By extending product shelf life, increasing productivity, and reducing labour requirements, dough freezing can speed up distribution to far-flung areas (Ban et al., 2016).

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Development of Biotechnological Protocols for the Valorization of Alternative Plant Matrices as a Strategy for the Sustainability of Agri-Food Systems

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This PhD thesis focused on the reuse of the food industry by-products using biotechnological approaches. In particular, i) red grape pomace and ii) flours obtained from carob pods without seeds were used to obtain yogurt-like prototypes and baked goods using selected microorganisms capable of inducing sensory, technological, nutritional and functional improvements.

Sviluppo di protocolli biotecnologici per la valorizzazione di matrici vegetali alternative come strategia per la sostenibilità dei sistemi agro alimentari

Questa tesi di dottorato ha riguardato il riutilizzo di sottoprodotti dell'industria alimentare attraverso l'uso di approcci biotecnologici. In particolare, i) vinacce derivanti da vinificazione in rosso e ii) sfarinati ottenuti da baccelli di carruba privati dei semi, sono stati utilizzati per ottenere bevande yogurt-like prototipali e prodotti da forno, utilizzando microrganismi selezionati in grado di indurre miglioramenti sia a livello sensoriale che tecnologico, nutrizionale e funzionale.

Key words: yogurt like, plant-based, bread, clean-label, biotechnological protocols, lactic acid bacteria.

1. Introduction

The Agenda 2030, signed on September 25, 2015, by the governments of the 193 member countries of the United Nations and approved by the UN General Assembly, consists of 17 Sustainable Development Goals (SDGs) to be achieved by 2030 in the environmental, economic, social, and institutional domains. The twelfth goal focuses on the implementation of sustainable production and consumption patterns. One of the implementation measures states, "Halve per capita global food waste at the retail and consumer levels and reduce food losses along production and supply chains, including post-harvest losses" (source: https://www.agenziacoesione.gov.it/comunicazione/agenda-2030-per-lo-sviluppo-sostenibile/). The utilization of by-products from the food industry, including carob pods without the seeds and grape pomace, is necessary to avoid wastage. In accordance with the PhD thesis project previously described, this oral communication reports the main results of the following three activities directed to:

A1) Use of grape pomace as an ingredient for fibre and antioxidant compounds fortification in bread
A2) Design of a plant-based yogurt like including red pomace grape-formulation and characterization of a new recipe
A3) Design of a high-fibre and "clean-label" plant-based yogurt like including carob flour -formulation and characterization of the new recipe

2. Materials and methods

2.1 Use of bioprocessed grape pomace as ingredient for fibre and antioxidant compounds fortification in bread

Once the composition of a pomace substrate had been optimized to be suitable for LAB (Lactic Acid Bacteria) fermentation, different starters, previously isolated from plant food matrices, were inoculated and incubated for 24 hours at 30°C: *Lactobacillus rossiae* T0A16, *Lactiplantibacillus plantarum* T0A10 and *Lactiplantibacillus plantarum* T6B10 isolated from quinoa flour (Rizzello et al., 2016) *Pediococcus acidilactici* 10MM0, *Lactiplantibacillus plantarum* 18S9 and *Leuconostoc mesenteroides* 12MM1 isolated from hemp (Nionelli et al., 2018), *Lactiplantibacillus plantarum* H22, *Pediococcus pentosaceus* H18 and *Lactiplantibacillus plantarum* H64 isolated from hops (Nionelli et al., 2018b), *Lactiplantibacillus plantarum* LB1 and *Lactobacillus rossiae* LB5 isolated from wheat germ and *Enterococcus faecium* CA16 isolated from carob. To select the best performing starter, total titratable acidity (TTA) values were measured before and after fermentation and lactic acid bacteria were enumerated by plate count. In addition, the pH of the optimized substrate was measured at defined time intervals during fermentation and the data were used to obtain acidification kinetics. Based on these results, the starter selected was used for obtaining type II sourdoughs (LNs), mixing wheat flour with grape pomace at 0% (LN0), 2.5% (LN2.5) and 5% (LN5) of the total weight of flour. The three LNs were incubated at 30 °C for 24 hours. Pre- (t0) and post-fermentation (t24) pH, TTA, total free amino acid (TFAA) concentration and

antioxidant activity, using the DPPH assay, were measured for each of the sourdoughs. At the end of fermentation, LAB were also enumerated, and lactic and acetic acid quantified. Based on the results obtained, the best sourdough was selected to be used for bread production. Three types of experimental breads were compared: control bread leavened with bakers' yeast (Ct LB), control bread with sourdough (Ct LN) and bread with the sourdough fortified with pomace (LNV). At the end of leavening, and thus before baking, volume increase, pH, TTA, acetic acid concentration and antioxidant activity were assessed. TFAA were quantified before and after baking using the automatic Biochrom 30+ Amino Acid Analyzer (De Pasquale et al., 2021). At the end of baking the structural characteristics of the loaves were determined, and the colour coordinates of the crust and crumb were measured. Finally, the sensory analysis of the breads produced was carried out.

2.2 Design and characterization of a yogurt-like and plant-based beverage fortified with red grape pomace

Red grape pomace, without separation of the grape seeds, was mixed with rice flour to obtain a yogurt-like (YL). Three different strains were then inoculated separately: *L. plantarum* T0A10, previously selected as able to grow in the pomace-enriched substrate and to increase antioxidant activity of plant-derived matrices (Rizzello et al., 2016); *Leuconostoc pseudomesenteroides* DMS20193, potentially able to produce exopolysaccharides in situ from sucrose (Montemurro et al., 2023); and *Lacticaseibacillus rhamnosus SP1*, a probiotic strain (Lorusso et al., 2018; Coda et al., 2011; Nionelli et al., 2014). An uninoculated control (Ct) was also prepared. After fermentation, the YL were characterized by enumerating the presumptive LAB and measuring their pH, TTA, viscosity, TFAA, organic acids, in vitro protein digestibility (IVPD), total polyphenols and antioxidant activity. A sensory analysis was also conducted for all fermented beverages. The analysis of pH, TTA, cell density and antioxidant activity were also repeated after 7 and 14 days of cold storage to assess the shelf-life of the fermented beverages.

2.3 Use of selected lactic acid bacteria and carob flour to produce a high-fibre and "clean label" plant-based yogurt-like

A new formulation of YL was designed using rice and carob flours. Six previously characterized LAB strains were used as starters to singly inoculate the rice/carob yogurt-like (YL). More specifically, *L. rhamnosus* SP1, chosen because already demonstrated optimal technological properties and high survival under refrigerated storage conditions (Lorusso et al., 2018; Coda et al., 2011; Nionelli et al., 2014), *L. plantarum T6B10* showed the best adaptation and highest acidification when used as starter in a quinoa YL (Lorusso et al., 2018), *Weissella cibaria* P9 and *L. pseudomesenteroides* DSM20193, chosen for their ability to produce exopolysaccharides (EPS) in several vegetable matrices (Montemurro et al., 2021; 2023) whereas *Levilactobacillus brevis* AM7 was selected due to its proteolytic activity and antimicrobial properties (Coda et al., 2008; Nionelli et al., 2020). Lastly, *E. faecium* CA16, isolated from carob pulp flour, was also used. A deep level of investigation in a case-by-case assessment should be provided before using such microorganisms as starters at industrial level (EFSA, 2007), since bacteria belonging to the genus Enterococcus are frequent opportunistic human pathogens and express adhesion factors, yet some strains are used as starter cultures in dairy products. At the end of fermentation cell densities of presumptive LAB, yeasts, molds, and enterobacteria were evaluated for all the YL. pH, TTA, organic acids, sugars, TFAA, water holding capacity (WHC), viscosity, and antioxidant activity were also determined. Biochemical, microbiological, technological, and functional characterizations were also repeated after 15 and 30 days of refrigerated storage to investigating the microbiological quality of the products and the survival of the inoculated strains under refrigerated conditions. Based on the previous results, the best rice/carob YL formulation was selected for subsequent nutritional and sensory analysis.

3. Results and discussion

3.1 Use of grape pomace as an ingredient for fibre and antioxidant compounds fortification in bread

Based on the ΔpH values, the 2,5 % LN supplemented with glucose and yeast extract was chosen to select the best starter for fermentation. At the end of fermentation, L. *plantarum* T0A10, T6B10, and LB1 exhibited a cell density higher than 9 log cfu/mL and greater antioxidant activity (70.63 ± 0.75%, 70.72 ± 1.80%, and 69.41 ± 3.40%, respectively). Among that strains, *L. plantarum* T0A10 was selected for further testing because it showed a short adaptation phase and, at the same time, a high final cell density, and antioxidant activity comparable to that of BHT (at 75 ppm). *L. plantarum* T0A10 was then used for obtaining type II sourdoughs added with 0% (LN0), 2.5% (LN2.5), and 5% (LN5) grape pomace. The starter was able to grow in all LNs. At the end of fermentation, the three different LNs had a pH of approximately 3.6. The antioxidant activity revealed that the addition of grape pomace already increased the antioxidant activity from about 7% (LN0) to 74% and 34% (LN5 and LN2.5, respectively) before fermentation. Fermentation further increased the antioxidant activity, which reached 89% and 48% respectively for LN5 and LN2.5 after 24 hours of incubation. An increase in TFAA was observed for all LNs compared to pre-fermentation values. The concentration of lactic acid and acetic acid was significantly lower in LN2.5 and LN5 compared to LN0. Based on these results, the LN enriched with 5% grape pomace was chosen for bread production (LNV). LNV was compared to control bread with bakers' yeast (Ct LB) and control bread with sourdough (Ct LN). During proofing, the volume increase of the three doughs bread

showed no significant differences. However, the final pH was lower in dough containing sourdough. The concentration of organic acids reflected what was found in the sourdoughs: the lactic acid in Ct LN and LNV was approximately 23-24 mmol/kg, with no significant differences between them, while acetic acid was significantly higher in Ct LN. Organic acids were only found in traces in Ct LB since both are associated with lactic fermentation (of *L. plantarum*) (Nuryana et al., 2019). The concentration of free amino acids was higher in bread containing LN compared to the control made with bakers' yeast. As expected, the antioxidant activity was markedly higher than in the two breads without grape pomace. The analysis of FAA showed that the addition of grape pomace alone led to an increase in Cys and Tyr. The nutritional label (Tab.1), commissioned to an external laboratory, showed that the three breads differ in fiber content, which is 30% higher in LNV compared to Ct-LB; fats and ashes are slightly but significantly higher compared to the two breads made with wheat flour alone; carbohydrates (excluding fibers) are slightly but significantly lower in LNV. However, the observed differences are not significant enough to determine a substantial variation in the energy value of the three types of breads. It is important to note that the increase in fiber content is attributed not only to the addition of grape pomace but also to the potential contribution from resistant starch generated due to the biological acidification associated with sourdough fermentation.

Table 1 *Nutrition labels of the experimental breads.*

	Ct LB	Ct LN	LNV
Moisture (%)	27.00 ± 1.00^{a}	27.00 ± 2.00^{a}	27.00 ± 1.00^{a}
Proteins (%)	8.68 ± 0.38^{a}	8.68 ± 0.48^{a}	8.71 ± 0.45^{a}
Fat (%)	0.87 ± 0.10^{b}	0.87 ± 0.08^{b}	0.95 ± 0.09^{a}
Carbohydrates (%)	61.63 ± 0.36^{a}	61.63 ± 0.42^{a}	61.05 ± 0.39^{b}
Fibres (%)	2.17 ± 0.14^{b}	2.27 ± 0.11^{b}	2.82 ± 0.19^{a}
Ashes (%)	0.52 ± 0.20^{b}	0.52 ± 0.10^{b}	0.60 ± 0.02^{a}
Energy (kcal)	289.00 ± 3.00^{a}	289.00 ± 2.00^{a}	287.00 ± 3.00^{a}
Energy (kJ)	1209.00 ± 13.00^{a}	1209.00 ± 6.00^{a}	1203.00 ± 7.00^{a}

$^{a-b}$ values marked with a different superscript are significantly different ($P<0.05$)

Structural analysis indicated that the breads had comparable hardness, although the use of sourdough, especially when supplemented with grape pomace, resulted in lower cohesiveness. In contrast to what is typically found in fiber-enriched breads, the bread containing grape pomace exhibited higher springiness, which is indicative of elasticity. The chewiness parameter suggested that LNV bread may require more effort for chewing.
The inclusion of grape pomace caused significant color variations. Both the crumb and crust showed a clear and notable reduction in lightness, with the crust exhibiting a significantly higher value of the "a" parameter (green/red) compared to the other breads. Based on evaluations by 10 tasters, the main differences between the sourdough bread (LNV) and the other two breads were primarily associated with the color of the crust and crumb, which appeared significantly more intense in the bread containing grape pomace. The differences related to the presence of sourdough involve aroma and acidic taste, which are typical attributes of sourdough breads and are associated with the metabolic activity of lactic bacteria. Among the positive notes, it is noteworthy that the friability of LNV bread was perceived like the control bred made with bakers' yeast, despite the higher fibers content. Astringency and herbaceous aroma/taste, attributes characteristic of grape pomace, were detected. However, the overall aroma of LNV bread received the highest rating among the three breads, indicating that it was not perceived as a defect.

3.2 Design and characterization of a yourt-like and plant-based beverage fortified with red grape pomace

At the end of fermentation, an increase in cell density of approximately 2 log cycles was observed for all the starters used (Tab. 2). In addition, intense acidification was observed, with lower pH values for DSM20193 (4.27) (Tab. 2). The lactic acid concentration was highest in DSM20193 and T0A10 (14.80 and 14.66 mmol/L, respectively). The latter also showed the highest concentration of acetic acid compared to the other samples (2.35 mmol/L) (Tab. 2). TFAA were analyzed at the end of fermentation to assess the proteolytic activity of the strains. A decrease in the concentration of free amino acids was observed in all samples (Tab. 2), with the greatest decrease for the DSM20193 sample (-62 %). The viscosity of Ct and SP1 was significantly similar (0.60 and 0.63 Pa x s, respectively), and lower than that measured for T0A10 and DSM20193. The latter, unlike expected, did not show particularly higher viscosity values than the other theses, suggesting no or limited exopolysaccharide synthesis under the test conditions (Tab. 2). A protocol mimicking *in vitro* digestion was used to estimate the IVPD of fermented beverages. It was observed that protein digestibility increased in all fermented samples compared to the control, especially in the sample fermented with T0A10 (49.59%) (Tab. 2). The total polyphenol concentration of the beverages was found to be in the range of 818-826 mg/l, with no significant differences between the samples (Tab. 2). The antioxidant activity of the three yogurt-like beverages, analyzed as radical scavenging activity on DPPH, is referred to a 1:3 dilution of the methanolic extract. The fermented theses all had significantly higher activity values than the control (Tab. 2). The results of the panel-test on the beverages shown that the fermentation reduced the perception of 'particulate' and 'earthy', which had higher

scores in the Ct sample. The perception of acidity, which characterized all fermented theses as an intrinsic characteristic of yogurt, was positively evaluated. A reduction in adhesiveness was also recorded in the fermented theses, probably related to the effect of acidification on the gelatinized starch, which leads to a progressive release of water from the samples. The fermented samples appeared to be relatively similar, except for the greater perception of sweetness in the DSM20193 sample, probably related to the amount of sucrose added and not fully converted by the starter into exopolysaccharides and organic acids. At the end of fermentation, the samples were kept at 4 °C and analyzed after 7 and 14 days of cold storage. In all samples, post-acidification was observed, which was particularly intense in DSM20193. It can be assumed that the addition of sucrose also favored increased lactic acid production during both production and cold storage. Cell density remained high and always exceeded 8 log cfu/ml. Antioxidant activity was persistent throughout the monitored storage period.

Table 2 *Characterization of the beverages at the end of incubation.*

	Ct	T0A10	SP1	DSM20193
Cell Density of LAB (log cfu/g)	2.70 ± 0.23^{b}	8.84 ± 0.04^{a}	8.56 ± 0.38^{a}	8.96 ± 0.04^{a}
pH	5.50 ± 0.00^{a}	4.39 ± 0.02 ^{b}	4.48 ± 0.01^{b}	4.27 ± 0.08 ^{b}
TTA (mL NaOH 0,1 M)	1.80 ± 0.15^{c}	4.20 ± 0.10^{a}	3.40 ± 0.15^{b}	4.60 ± 0.07^{a}
Lactic acid (mmol/L)	0.66 ± 0.00^{c}	14.66 ± 0.01^{a}	11.49 ± 0.02^{b}	14.80 ± 0.03 ^{a}
Acetic acid (mmol/L)	0.45 ± 0.01^{c}	2.35 ± 0.01^{a}	0.59 ± 0.02^{b}	0.69 ± 0.05^{b}
Free Total Aminoacid (mg/L)	65.46 ± 3.44^{a}	35.82 ± 7.71 ^{c}	48.53 ± 8.50 ^{b}	24.94 ± 1.26^{d}
Viscosity (Pa x s)	0.60 ± 0.02^{b}	0.70 ± 0.02^{a}	0.63 ± 0.04^{b}	0.67 ± 0.01^{a}
Total Phenols (mg/L)	826.00 ± 49.49^{a}	819.00 ± 53.27 ^{a}	823.00 ± 46.64 ^{a}	818.00± 42.67 ^{a}
Radical Scavenging Activity on DPPH*	50.09 ± 0.03^{c}	58.77 ± 0.02^{a}	52.54 ± 0.03^{b}	53.94 ± 0.02^{b}
In vitro protein digestibility (%)	28.69 ± 8.36^{c}	49.59 ± 7.21^{a}	47.56 ± 4.88^{b}	40.99 ± 11.24^{b}

*samples diluted 1:3
$^{a-d}$ values marked with a different superscript are significantly different (P<0.05)

3.3 Use of selected lactic acid bacteria and carob flour to produce a high-fibre and "clean label" plant-based yogurt-like

The pH of the rice-carob mixture employed as substrate for fermentation ranged from 5.5 to 5.6 (Tab. 3). After 16h of fermentation, all samples had pH values lower than 5.0, with only T6B10 reaching a pH lower than 4.5 (4.33). Despite thermal treatment, the rice-carob gelatinized mixture still contained low Enterobacteriaceae, yeasts and moulds. During incubation, all inoculated strains increased by approximately 2 log cycles, with cell densities ranging from 9.00 to 9.73, at the end of fermentation (tf). Moreover, Enterobacteriacee decreased significantly in chemically acidified control (Ct) and in YL fermented with DSM20193, SP1, T6B10 and AM7 compared to t0. Molds were not detected in inoculated samples at tf. The lactic acid concentration in all YL after fermentation ranged from 7.21-8.80 mmol/L with T6B10 having the highest concentration (20.17 mmol/L, Tab. 3). Acetic acid concentrations were low in all inoculated samples, with higher levels observed in P9 and 20193 (Tab. 3).

Table 3 *Main characteristics of the YL after incubation at 30°C for 16h (tf). A control sample (Ct) corresponding to a not inoculated, but chemically acidified YL, was also characterized.*

	Ct	**CA16**	**DSM20193**	**SP1**	**T6B10**	**AM7**	**P9**
pH	4.50^{c}	4.91^{b}	4.56^{c}	4.71^{b}	4.33^{d}	4.68^{b}	4.87^{b}
TTA (mL NaOH 0,1 M)	3.40^{b}	3.00^{c}	4.00^{a}	3.60^{b}	4.40^{a}	3.40^{b}	3.00^{b}
Lactic acid (mmol/L)	12.07^{c}	7.21^{d}	11.69^{c}	13.54^{b}	16.17^{a}	13.98^{b}	8.80^{d}
Acetic Acid (mmol/L)	n.d.	0.21^{d}	1.28^{a}	0.65^{b}	0.50^{c}	0.61^{b}	1.36^{a}
Glucose (g/L)	1.66^{a}	1.01^{b}	0.43^{d}	0.47^{d}	0.54^{d}	0.80^{c}	0.45^{d}
Fructose (g/L)	2.14^{c}	2.41^{b}	2.79^{a}	2.53^{b}	2.83^{a}	2.44^{b}	2.57^{b}
Maltose (g/L)	0.52^{a}	0.45^{a}	0.46^{a}	0.50^{a}	0.56^{a}	0.50^{a}	0.55^{a}
Sucrose (g/L)	16.86^{a}	17.04^{a}	16.06^{c}	16.02^{c}	15.85^{c}	16.47^{b}	16.84^{a}
Total Free Amino Acid (mg/L)	159.00^{a}	76,00^{c}	113.00^{b}	159.00^{a}	112.00^{b}	123.00^{b}	156.00^{a}
WHC (%)	82.26^{c}	85.82^{c}	93.88^{a}	90.59^{b}	91.20^{b}	84.41^{c}	84.16^{c}
Viscosity (mPa x s)	8180.00^{d}	12760.00^{b}	9920,00^{c}	9720.00^{c}	9770.00^{c}	9800.00^{c}	12020.00^{b}
Radical scavenging activity	81.96^{a}	81.08^{a}	82.23^{a}	81.62^{a}	82.90^{a}	82.67^{a}	83.08^{a}

Nd = not detected
$^{a-d}$ values marked with a different superscript are significantly different (P<0.05)

Glucose concentration significantly decreased in all inoculated samples (-50/70%), except for CA16 (Tab. 3). Significant decreases were also found for sucrose in AM7, SP1, DSM20193, and especially T6B10 (Tab. 3). No variation was observed in maltose concentration during incubation, while fructose concentration slightly increased in inoculated samples, particularly in DSM20193 and T6B10 (Tab. 3). Before fermentation, the yogurt-like substrate had a total TFAA concentration ranging from 159 to 163 mg/L (Tab. 3). Significant

decreases in TFAA occurred during fermentation in CA16, T6B10, 20193, and AM7, while no significant decreases were found in SP1 and P9 (Tab. 3). The antioxidant activity was already high at the start of fermentation and remained unaffected by the process (Tab. 3). WHC increased significantly in three out of six inoculated samples (DSM20193, T6B10, and SP1, Tab. 3). Viscosity data showed a correlation with acidification during fermentation, with only CA16 and P9 having higher values than 12000 mPa x s at the end of fermentation. The other samples experienced decreases of approximately 30% compared to their initial values (Tab. 3). At the end of the 30 days of refrigerated storage, LAB cell density in YL decreased of ca. 1 log cfu/mL. Yeasts persisted in all the inoculated YL samples, while a significant increase was observed in Ct. No Enterobacteriaceae and molds were found in any samples. Furthermore, all samples showed a slight but significant decrease in pH compared to t15, accompanied by increases in lactic and acetic acids. A further decrease of sucrose also occurred. Regarding the TFAA, a significant increase characterized T6B10 (+87 and +46% compared to tf and t15 respectively). A significant ($P<0.05$) increase in TFAA was also observed in DSM20193 (+41% compared to tf and t15), although final concentration was markedly lower than that found for T6B10.

Based on these results, *L. plantarum* T6B10 was identified as the starter capable of the most intense and fast acidification of the substrate. Additionally, the YL-T6B10 was characterized by the highest concentration and better-balanced mixture of FAA. For these reasons YL-T6B10 was further characterized for its nutritional and sensory profiles. YL-T6B10 had the following nutritional label: proteins, 1.58 g/100g; fats, 0.36 g/100g; carbohydrates 15,41 g/100g of which 2.40 sugars; and 2.66 g/100g dietary fibres). The energy value was 76.34 Kcal/100g. The starch hydrolysis index (HI) of the two samples analysed were 53.47 ± 3.45 and 37,99 ± 1,01 % respectively for Ct and T6B10. Consequently, the pGI of the Ct was markedly and significantly ($P<0.05$) higher than that of T6B10 (69.07 ± 1,89 vs 60.57 ± 0,91).

Aiming at describing the sensory profile of the YL-T6B10 and highlighting the effect of the fermentation, a list of descriptors was selected during the preliminary sessions of the analysis. YL-T6B10 appearance was characterized by a very high score for uniformity and adherence to spoon. For both the attributes, values were significantly higher than control (chemically acidified substrate). Odour intensity and cocoa smell were also intensified by the fermentation. Sweet and bitter taste perceptions were lower in fermented samples compared to control, and astringent and earthy aftertaste resulted significantly lower in YL-T6B10.

4. Conclusions and Future Perspectives

These studies demonstrated the suitability of bioprocessed food industry by-products such as carob and grape pomace, to be used as ingredients in the formulation of innovative plant-based foods, such as yogurt-like and baked goods. Moreover, the results may be considered as the basis for future research for the development of novel ingredients through the use of lactic acid bacteria strains with specific functional features.

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Modifications of vegetables subjected to conventional, innovative and non-thermal technologies

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This study deals with the HPP treatment with different pressures and time profiles on vegetables to determine the physio-chemical, microstructural, antioxidant, bioactive, volatile and sugar content of pumpkin samples; subsequently, it is fundamental to explore and see how this technology impacts the vegetable characteristics.

Analisi delle modificazioni nella microstruttura di vegetali sottoposti a tecnologie convenzionali, innovative e non termiche

Questo studio si occupa del trattamento HPP con diverse pressioni e profili temporali su ortaggi per determinare il contenuto fisico-chimico, microstrutturale, antiossidante, bioattivo, volatile e zuccherino di campioni di zucca; successivamente, è fondamentale esplorare e vedere come questa tecnologia influisce sulle caratteristiche vegetali.

Keywords: HPP, Pumpkin, color-texture, Histological analysis, bioactive and volatile components

1. Introduction

Food must be treated in order to extend its shelf life because it is often a perishable commodity. Thermal processing is an effective method for microbial inactivation, although it has drawbacks and side effects. This opens up a field for the investigation of non-thermal processing methods. High-pressure processing (HPP) is a non-thermal technique for food preservation with little loss in quality. The process involves the application of high pressures (up to 1000 MPa) for a short time (Chauhan et al., 2019) with the aim to reduce food pathogens at room temperature, to inactivate the deteriorative enzymes, to extend the shelf life (Zhou et al., 2014) with little impact on the nutritional and chemical composition of foods. HPP technology was used in fruit and vegetable products; results indicate an impact on texture, color and flavor, but the intensity of the changes depends on both process conditions and the type of plant tissue treated (Oey et al., 2008). From the anatomical point of view, HPP influences the microstructure (cells and tissues) too: there are many changes at the cellular and tissue level such as changes in cell shape due to loss of turgor, damage to the cell wall and membrane and formation of cracks in the plant tissues (Trejo Araya et al., 2007; Contador et al., 2014; Paciulli et al., 2021). All these changes are responsible for the loss of firmness of fruits or vegetables. In addition, Dhenge et al. (2022) observed that HPP technology has the potential for stability and availability of all bioactive components, volatiles and sugars. Thus, HPP might be used as a substitute method to improve the effectiveness of bioactive component extraction (e.g., phenolic, carotenoids, volatile compounds). In any case, the application of high-pressure processing on juices, paste, and purees is not quite the same as to whole pieces of vegetables; subsequently, it is fundamental to explore and see how this technology impacts the fruits and vegetable characteristics**.** The aim of this study is to assess the effects of HPP of selective pressures and time on pumpkin samples.

2. Materials and Methods

Pumpkin (*Cucurbita moschata* Duchesne ex Poir) samples were cut into small cubes from 1 to 1.5 cm (UNTR) and then vacuum packed in HDPE bags and subjected to high-pressure processing of selective pressures such as 200, 400 and 600 MPa for 1(A), 3(B) and 5(C) mins at 20°C. After the treatment, all samples were stored at 4°C, next day samples were used for the physical (color by Konica Minolta and texture by texture analyzer using a TA. XT2i) and chemical analyses (polyphenols and carotenoids (LC-MS)), volatiles (HS-GC-MS), sugars (HPLC) and antioxidant capacity (DPPH)). For the microstructure examination, the fixed and dyed sections were observed by means of an optical microscope at different magnifications (5, 10, 20, 40 and 63X). For the evaluation of the general structure variation, the sections were stained with toluidine blue (TBO) solution and to identify the presence of calcium inclusions in tissue sections, the sections were stained by Von Kossa (Bio-Optica Kit, Milano, Italy).

3. Results and Discussion

3.1 Physical analysis

Moisture, total soluble solids (TSS) and pH were measured. The Values of pH ranged from 6.03 to 6.60, the values of total sugar content ranged from 8.5 to 10.86 °Brix, while the moisture content ranged from 85.40 to 87.66 (g/100g). The data obtained showed statistically significant differences among samples.

3.2 Colorimetric analysis

In the **UNTR** sample, all colorimetric values (*L**, *a** and *b**) were the highest with a great color difference compared to HPP ones. A significant difference was observed from all pressure samples whereas **HPP400B, 600A** and **600B** showed little greater color values than other HPP-treated samples (Fig. 1). This might be caused by cell disruption during HPP treatment resulting in the leakage of pigment into the intercellular space or degradation of pigment yielding a less intense color. A significant change was induced when pressures above HPP400 were applied, showing that, in our condition, extra pressure may not be a good choice for vegetable processing.

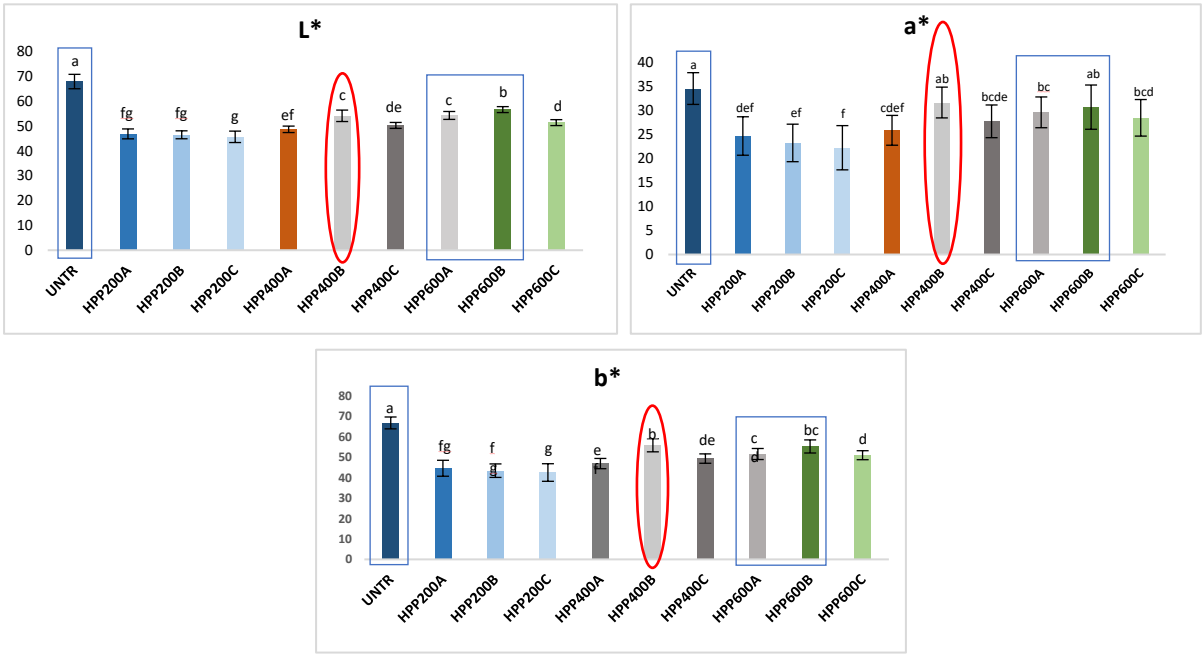

Figure 1 *Colorimetric analysis of UNTR and HPP-treated pumpkin samples.* ***Letters****:* ***UNTR****: raw sample,* ***HPP****: pressure treated samples (200, 400 and 600MPa),* ***A*** *(1mins),* ***B*** *(3 mins) and* ***C*** *(5 mins).*

3.3 Texture profile

A noticeable difference was observed in **HPP400B** and **600C** samples, with a difference in terms of hardness (155.26 and 161.05 ± 3N) compared to the UNTR ones (312.96 ± 46.3 N) whereas treatments at other pressures changed the texture of the pumpkin samples, but less markedly (Table 1). The softening of the texture and decrease in the hardness of plant tissue is caused by cell wall breakdown, cell rupture, degradation of pectin and loss of turgor pressure induced by high pressure. As pressure increases, hardness decreases and enhances the activity of PME (Oey et al., 2008) which has a substantial impact on cell damage, breakdown of cell walls' structure and release of pectin and calcium, and less adhesiveness between the cell and cell dehydration. Other textural characteristics such as resilience, cohesiveness, springiness, and chewiness indicate better texture quality retention in UNTR than HPP treated samples.

Table 1 *Textural parameters of UNTR and HPP-treated pumpkin samples.* ***Letters****:* ***UNTR****: raw sample,* ***HPP****: pressure treated samples (200, 400 and 600MPa),* ***A*** *(1mins),* ***B*** *(3 mins) and* ***C*** *(5 mins).*

Treatment	Hardness (N)	Resilience (%)	Cohesiveness	Springiness (%)	Chewiness
UNTR	**312.96±46.3a**	**32.51±2.0a**	**0.58±0.03a**	**58.37±3.2a**	**108.53±23.4a**
HPP200A	227.33±26.0b	22.01±7.0bcd	0.37±0.1bcd	57.37±13.5a	47.60±11.3bc
HPP200B	232.55±43.1b	25.24±8.5ab	0.42±0.1b	52.31±3.0ab	52.61±21.6b
HPP200C	224.43±41.6b	25.05±5.8abc	0.42±0.08cd	52.43±3.7ab	50.99±17.7b
HPP400A	189.68±54.7bc	19.20±4.9bcde	0.31±0.07cdef	44.85±5.7b	28.86±15.5cde
HPP400B	**155.26±38.3c**	**13.24±4.2e**	**0.23±0.05f**	**46.88±11.2b**	**17.67±8.7e**
HPP400C	206.15±47.9bc	21.18±6.6bcd	0.35±0.1bcde	50.47±9.7ab	37.74±17.2bcde
HPP600A	210.42±53.5bc	21.51±6.7bcd	0.35±0.09bcde	50.31±5.2ab	39.08±17.2bcd
HPP600B	183.3±48.1bc	17.48±3.9cde	0.27±0.05def	45.50±4.6b	24.41±11.2de
HPP600C	**161.05±38.0c**	**15.70±4.8de**	**0.25±0.06ef**	**45.60±5.4b**	**19.13±8.6de**

3.4 Microstructure analysis

The microstructure of pumpkin samples appeared to be changed after HPP treatments. In the untreated samples (UNTR), the inner parenchyma (mesocarp) is composed of isodiametric cells with thin cell walls. Mesocarp is composed of thin-walled big and small cells with large intercellular spaces (is) (Fig. 2A). In the inner parenchyma, vascular bundles (vb) are present and its surrounded by small parenchymatic cells. Pumpkin tissue showed great structural modifications such as changes in cell size and shape, cell wall damage, increases cell wall thickness, cell detachment, cell dehydration and calcium ions deposition mainly at very high pressures (400C, 600A and 600C) **(**Fig. 2D-E-F**)** (Trejo Araya et al., 2007; Zhou et al., 2014). UNTR samples showed the highest value of maximum and minimum cell elongation, perimeter segment and more regular cell size -shape, cell wall thickness and a higher degree of cell-to-cell contact throughout the tissue whereas HPP 400C, 600A and 600C samples showed the lowest values for the same parameters (Zhang et al., 2015).

Another impact was observed regarding the calcium ion (ci) deposition in tissues. UNTR and HPP200 samples showed a scarce presence of calcium inclusions, but after the HPP400, the number of calcium inclusions (ci) increased (Fig. 3C–D). More interesting results about high calcium ions (ci) deposition were observed in HPP600A and 600C (Fig. 3E-F) mainly due to the liberation of calcium from the middle lamella which is previously bound in the pectin network. Our study reveals that as pressure increased, the presence of calcium ions in the cells increased and was liberated as a result of cell separation.

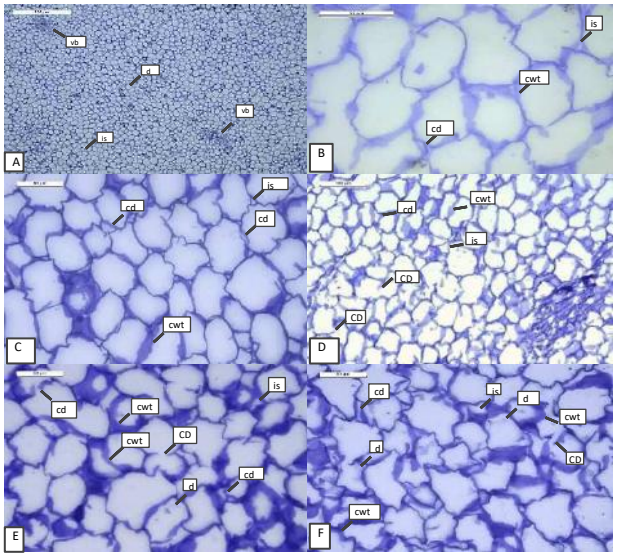

Figure 2 *Light microscopy images of Violina Squash stained with TBO (Toluidine Blue) (A, B, C, D, E, and F): A-200**A** (1mins); B-200**C** (5mins); C-400**A** (1mins); D-400**C** (5mins); E-600**A** (1mins); F-600**C** (5mins). Legends: vb = vascular bundles; is = intercellular space; d = dehydration, cd = cell detachment; CD = cell damage; cwt = cell wall thickness (increase). Letters:**A** (1mins), **B** (3 mins) and **C** (5 mins).*

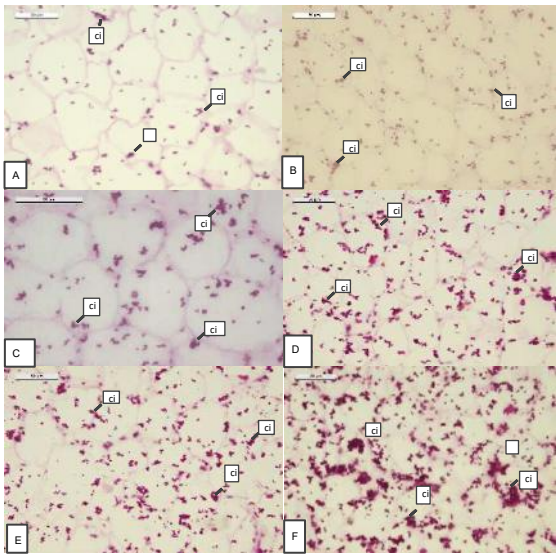

Figure 3 *Light microscopy images of Violina Squash stained with Von Kossa (Bio-Optica kit, Milano) (A, B, C, D, E, and F): A-200**A** (1mins); B-200**C** (5mins); C-400**A** (1mins); D-400**C** (5mins); E-600**A** (1mins); F-600**C** (5mins) . Legends: ci = calcium ions. Letters: **A** (1mins), **B** (3 mins) and **C** (5 mins).*

3.5 Antioxidants and bioactive components availability

Antioxidant capacity (AC): The HPP400A reported the highest equivalent value. On the contrary, the HPP significantly reduced the AC for HPP200B, 600B and 600C samples whereas HPP200C and HPP600A showed greater values but the highest AC observed in HPP400A comparison to other samples.

Table 2 *Antioxidant capacity by DPPH **Trolox eq. mmol/g dry wt. Basis** of UNTR and HPP-treated pumpkin samples. **Letters**: **UNTR**: raw sample, **HPP**: pressure treated samples (200, 400 and 600MPa), **A** (1mins), **B** (3 mins) and **C** (5 mins).*

	UNTR	HPP200A	HPP200B	HPP200C	HPP400A	HPP400B	HPP400C	HPP600A	HPP600B	HPP600C
Pumpkin samples	4.86±0.90^{d}	10.88±2.30^{abc}	6.45±1.20^{cd}	12.8±4.18^{ab}	15.14±1.96^{a}	8.59±0.99^{bcd}	11.91±2.25^{abc}	13.09±1.25^{ab}	6.67±0.31^{cd}	4.92±0.45^{d}

Polyphenols: Three main polyphenol derivatives (Luteolin-7-o-glucoside, 2-Rhamnosyl-glucosyl kaempferol, and Laempferol-7-o-glucoside) were detected by extraction and by squeezed. The Higher number of polyphenols was obtained in the sample from HPP400 (A and C) than in HPP600 (B and C). The content of polyphenol was higher in HPP400A its 40 mg/kg compared to HPP600. This is indicated that HPP at middle pressure like 400 At less time can have an influence on the polyphenol's composition and composition of the extractable polyphenols from the matrix. The concentration of the squeezed number of polyphenols for HPP**400C** obtained significantly higher (275 mg/kg) compared to others. For 200B, the squeezable number of polyphenols was very similar to the concentration obtained for HPP600B.

Carotenoids: A higher number of extractable total carotenoids was observed at higher pressure (**HPP600C**), whereas at middle pressure was also observed higher total carotenoids content (**HPP400 A and C and 600B**) than UNTR (Fig.4). Our results show that pumpkin is a rich source of carotenoids, especially β-carotene, lutein and other derivatives, and these derivatives might be increased at moderate pressure, because at increased pressure oxidative chemical reaction was enhanced which is responsible for carotenoids degradation.

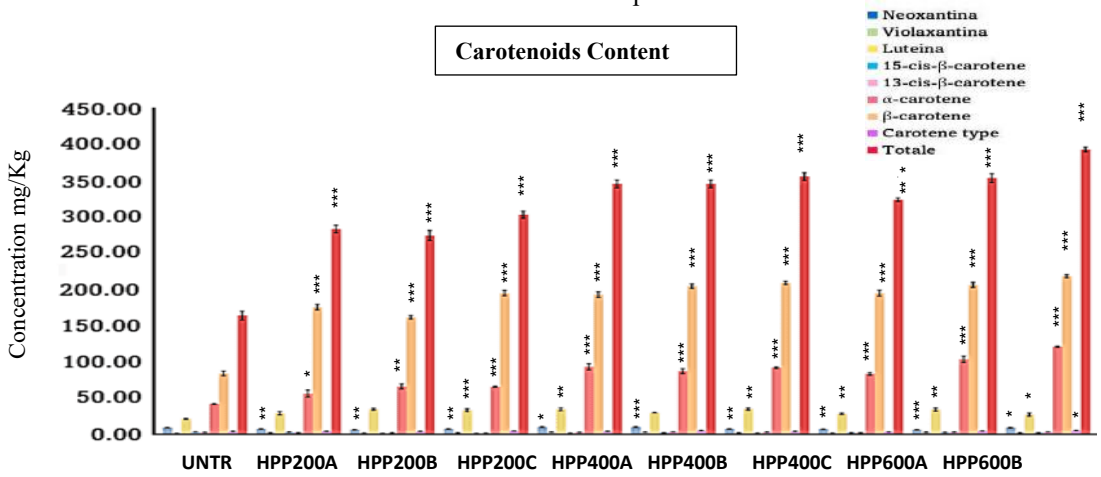

Figure 4 *Carotenoids content of UNTR and HPP-treated pumpkin samples. **Letters**: **UNTR**: raw sample, **HPP**: pressure treated samples (200, 400 and 600MPa), **A** (1mins), **B** (3 mins) and **C** (5 mins).*

3.6 Volatile and sugar profile

Regarding **volatile compounds**, some compounds were identified such as 3-Hexanol (1), 4- Hydroxy-3-Hexanone (2), 2-Methylfuran (3), 1,1-Dimethoxypropane (4), 2-Pentylfuran (5) and 1-Hexanol (6) respectively. Significant differences were observed between treated versus untreated samples. The Higher number of total volatiles was obtained in the sample of HPP400A, 600B and 600C than HPP200 and UNTR (Fig. 5). The Higher number of **total sugars** was obtained in the sample from HPP400C than in UNTR and HPP600. The content of sugar was also showed higher in HPP200C and HPP400B compared to other samples (Fig. 6). This is indicated that HPP at middle pressure like 400 at little more time can have an influence on the sugar composition but it decreased with high pressure treatment (HPP600) because of the more leaching effect.

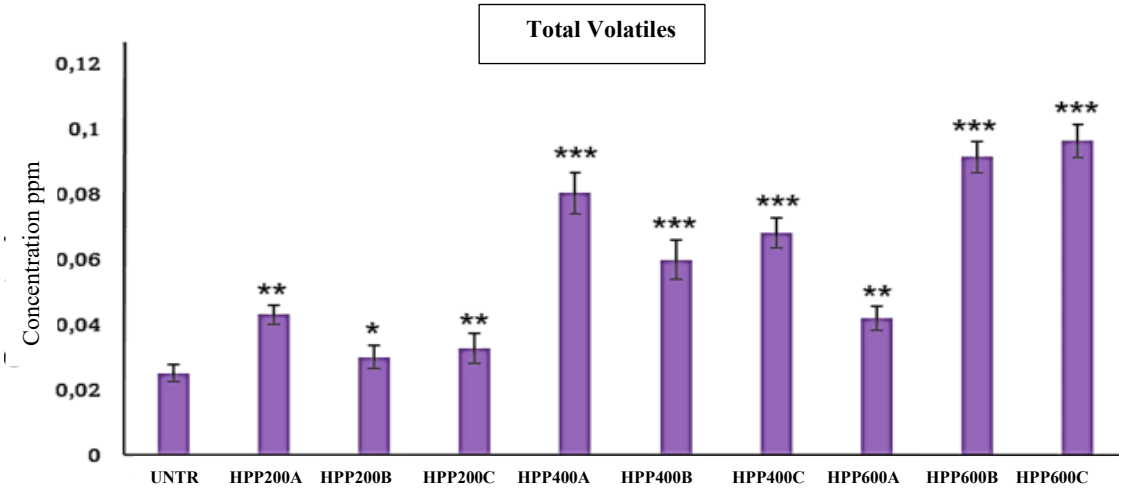

Figure 5 *The graph shows the concentration in ppm and standard deviation of volatiles identified in untreated and treated pumpkin samples. **Letters**: **UNTR**: raw sample, **HPP**: pressure treated samples (200, 400 and 600MPa), **A** (1mins), **B** (3 mins) and **C** (5 mins). * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).*

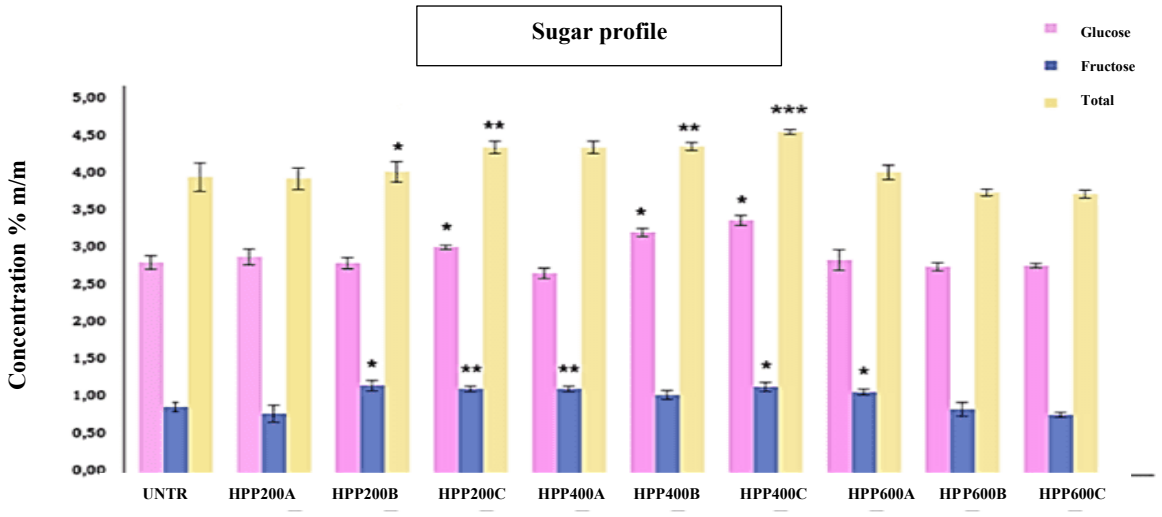

Figure 6 *Concentration in % m/m and standard deviation of glucose, fructose and total sugars in untreated and treated samples.* ***Letters****:* ***UNTR****: raw sample,* ***HPP****: pressure treated samples (200, 400 and 600MPa),* ***A*** *(1mins),* ***B*** *(3 mins) and* ***C*** *(5 mins). * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).*

4. Conclusion

The high-pressure processing (HPP) is a valuable approach for treating foods because it allows preservation without additives or heat. In this work, we have considered pumpkin as model food and evaluated the effects of HPP process at different pressures and time profile on different structural and chemical constituents. In particular, Untreated samples didn't show a colour, textural loss and microstructural changes. Pumpkin tissue showed great structural modifications such as changes in cell size and shape, cell wall damage, increases cell wall thickness, cell detachment, cell dehydration, and calcium ions deposition mainly at very high pressures and more time (HPP400C, 600A, B and C). High-pressure treatment from HPP200 to 400 (A and B) pressure less markedly influenced the structural quality means texture and microstructure then others. On the contrary, HPP400A showed a higher amount of antioxidants components availability. On the basis of these results data, we conclude that the treatment with intermediate pressure could ensure a higher amount of "availability" of polyphenols, carotenoids, volatiles, and total sugars in pumpkin sample. In the present study from the reported investigation we understood that the HPP at moderate pressure levels seems to be suitable for retaining stability and concentration of all some quality characterstics and additionally, its acceptable for commercial application for fresh pumpkin.

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Food Products and Microplastics: A Call for Qualification and Quantification

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This PhD thesis dealt with the assessment of microplastic's contamination of some food products by developing an analytical methodology for the pre-treatment of the food matrices by evaluating digestion efficiencies as well as the impact of digestion agents on microplastics. Furthermore, qualification and quantification of microplastics by means of pyrolysis – gas chromatography – mass spectrometry (Py-GC-MS) has been conducted.

Alimenti e Microplastiche: Una Necessaria Qualificazione e Quantificazione

Questa tesi di dottorato ha trattato la valutazione della contaminazione da microplastica di alcuni prodotti alimentari sviluppando una metodologia analitica per il pretrattamento delle matrici alimentari valutando l'efficienza della digestione e l'impatto degli agenti di digestione sulle microplastiche. Inoltre, sono state condotte la qualificazione e la quantificazione delle microplastiche mediante pirolisi - gascromatografia - spettrometria di massa (Py-GC-MS).

Key words: Microplastics; foods; pollution; digestion; pyrolysis – gas chromatography – mass spectrometry;

1. Introduction

In accordance with the PhD thesis project previously described, this oral communication reports the main results of the following four activities directed to:

A1) investigation of the best experimental conditions (i.e., time/temperature, sample/digestion reagent ratio) for digestion of organic matter of foods, necessary for visually analyse microplastics shape, color, size;
A2) assess the impact of digestion agents on microplastic particles, in terms of shape, size and color;
A3) determination of microplastic's recovery rate;
A4) solubilization of plastic polymers for quantification of microplastics using Py-GC-MS;

2. Digestion Efficiency and Quantification of Microplastics

Digestion efficiency (DE) is the parameters used for assessing the percentage of organic matter removed. Digestions are generally required in microplastic's research field, especially when the matrix is rich in biological materials (e.g., food products) (Prata *et al*., 2019). Isolation of microplastics from matrices still poses a major challenge, but its optimization is necessary to identify microplastics in a sample (Pfeiffer *et al*., 2020). Digestions have been applied to fish for human consumption, but experimental parameters (i.e., time/temperature, sample/digestion reagent ratio) (Table 1) varied among the different research's group (Makhdoumi *et al*., 2023).

Table 1 *Experimental conditions applied to fish for human consumption*.

Sample	Experimental conditions
Edible tissues	Potassium hydroxide 10% at 60°C 24 h (Daniel *et al*., 2020)
	Potassium hydroxide 10% at 50°C 48 h (Mistri *et al*., 2022)
	Hydrogen peroxide 30% 65°C 24-48h (Li *et al*., 2015)
Sample/reagents ratio	
	N/A (Daniel *et al*., 2020)
	N/A (Mistri *et al*., 2022)
	N/A (Li *et al*., 2015)

Papers which aim to assess the occurrence of microplastics in seafood do not carefully investigate the digestions conditions as well as data on digestion efficiencies achieved are not reported. Digestion reaction are influenced by the temperature (Karami *et al*., 2017). Likewise, optimizing the sample-to-reagent ratio can reduce the time needed for digestion and minimize the potential damage to microplastics from the digestion agent. This highlights the importance of identifying the best experimental conditions. Other food matrices (such as pasta, meat and cheese, mozzarella) are not documented in scientific literature in terms of occurrence of microplastics and so, digestion protocols. Scientific hypothesis seems to suggest the contamination of these foods. Particularly, Food and Agriculture Organization (FAO) suggested that plastic-packaging is a remarkable source of microplastics in foods (Gamarro and Costanzo, 2022). Optimisation of sample preparations is fundamental to assess microplastic's contamination in foods and so, for assessing the recovery rate (Cai *et al.,* 2019). These treatments should be standardised to obtain results as comparable as possible among researchers' groups.

Another important issue in microplastic's research field is their quantification. Currently, visual counting is the most used (Hamed *et al.,* 2019; Zeytin *et al.,* 2020). However, quantification by pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) is now a promising technique but, issues are still challenging. For example, for quantifying microplastics, the construction of calibration curves is required (Ishimura *et al.,* 2020). Furthermore, for food, where the contamination is supposed to be in the range of ppt, the method should be very sensitive. Therefore, for achieving low concentration in the calibration curves 'construction, solubilization of polymers is a potential solution. Hansen Solubility Parameters were taken into account for studying the solubilization of plastic polymers. The theory as well as formula are based on the interactions between solvent and plastics polymers. Briefly, the degree of similarity between solvent and solute, in a given situation, determines the extent of interaction. The key factor, in fact, of this theory is to identify the affinities that the components of a system have among themselves. The Hansen parameters (i.e., δ^d, δ^p, δ^h) indicate specific characteristics of the polymer. Whether a solvent is capable of dissolving a polymer is indicated by the *Ra/R0* ratio. Specifically, if the ratio *Ra< R0*, then the probability that the solvent will dissolve the polymer is high.

3. Mathematical Modelling

To design and optimise digestion efficiency, several parameters are be taken into account, such as time, temperature, type of agents, sample/reagent ratio.
Parameters were experimentally evaluated, to assess the best condition. Digestion efficiency (and the related standard deviation for each protocol) were assess with the following formula (1).

$$DE(\%) = 100 - \frac{Dry\ weight\ on\ filter\ paper\ (g)}{Wet\ weight\ entire\ matrix\ (g)} * 100; => \frac{W_i - (W_a - W_b)}{W_i} * 100 \quad (1)$$

Furthermore, percentage recovery rate (RR) of microplastics were assessed, using the following formula (2). where all symbols are given in section 7.

$$RR\ (\%) = \frac{N_a}{N_b} * 100 \quad (2)$$

Regarding the solubilization of plastics polymers for the construction of calibrations curves, Hansen Solubility Parameters were taken into account (3).

$$R_a{}^2 = 4(\delta_{d2} - \delta_{d1})^2 + (\delta_{p2} - \delta_{p1})^2 + (\delta_{h2} - \delta_{h1})^2 \quad (3)$$

which was calculated for each tested organic solvent, whereas for each polymer is experimentally determined.
For assessing whether a solvent was potentially able to dissolve a plastic polymer, the following formula was used:

$$\frac{R_a}{R_0} \quad (4)$$

If the ratio (4) was minor than 1, there were good possibility that the solvent dissolved the polymer.
All symbols used in this section are given in section 7.

4. Experimental Procedure

In this PhD thesis several experiments were conducted for the assessment of the best experimental design for each food matrix. Precisely, for fish samples, the type of reagent was not investigated as KOH was the most used. Therefore, parameters such as sample-to-reagent ratio, temperatures, digestion efficiency were studied. On the contrary, for pasta, and meat, all parameters were tested, such as type of reagent for the digestion, sample-to-reagent ratio, temperature and digestion efficiency were evaluated. In this way, it was possible to identify the best condition for each matrix. Afterwards, microplastic's percentage recovery rate was assessed, by visual counting. Recovery rate was determined to determine whether conditions used for digestion are able to clearly quantify microplastics. Regarding the preliminary solubility tests, solubilisation of powered polymer was tested using organic solvents suggested by the Hansen Solubility Theory, for each polymer. For quantification of microplastics by assessing the mass content, dissolved polymers were spiked in a known concentration on real samples and digested. After that, filters were analysed by Py-GC-MS. Pyrolysis products were used for qualification of microplastics, as well as for quantify them by a comparison with calibration curves.

5. Materials and Methods

For the digestion of organic matter, digestion solutions were prepared. Briefly, to ensure the absence of contamination, experimental solutions were prepared under a fume hood and workers wore blue nitrile gloves and cotton lab coat. Glassware used for the experiments were carefully washed and rinsed with MilliQ water, prior to use. Furthermore, solutions were filtered used filter paper for ensuring the absence of microplastic particles from the glassware or the air. Stirring-plates were used for digesting organic matter. For evaluating the best sample/reagent ratio, for each matrix, three rations were tested, such as 1:20, 1:40 and 1:60, using 0.5 g of food and 10, 20 and 30 mL of digestion reagent. Temperatures tested were 50°C and room temperature. For

testing the best type of digestion reagent, experiments were conducted in triplicate. For the assessment of the dissolution of plastic polymers into organic solvents, analytical grade solvents (> 98%) were used. Powered polymers such as polystyrene (PS, 150 µm), polyethylene terephthalate (PET, 300 µm) and polyethylene (PE, 150 µm) (Goodfellow, UK) were used. Therefore, polymers were weighted (0.005 g) and placed into a clean bottle. Afterwards, appropriate solvent (5 mL) (or mixture of solvents) was added and the solution was held in rotation for 24h at room temperature. Briefly, for PS, dichloromethane (DCM), chloroform, toluene, tetrahydrofuran (THF) and mixture of DCM: toluene (1:1; 1:2; 2:1) and DCM: *n*-hexane (1:1; 1:2; 2:1) were tested as were suggested by the theory. Likewise, THF: DMC (1:1; 1:2; 2:1) and hexafluoro2-propanol (HFIP) (even though the latter was not suggested by the theory) were evaluated. For PE, the theory suggested a mixture of n-hexane: toluene (1:1; 1:2; 2:1). For investigating the recovery rate of microplastics, a solution of fluorescent microbeads (diameter,10 µm) was used. About 10 µL of the dilute solution 1/1000 were spiked on the samples. Microbeads were counted thrice using a visual microscope. Afterwards, samples were digested with the appropriate techniques for each matrix analysed. The solution was filtered (Whatman, pore size 1.6 µm) and the recovered particles were counted thrice. For a preliminary quantification of microplastics using Py-GC-MS, samples were spiked with a known concentration (100 ppb) of the polymer solution. Samples were digested and filtered. Filters were analysed by Py-GC-MS.

5. Results and Discussion

5.1 Digestion Efficiency

Digestion efficiencies for matrices analysed are reported in Table 2.

Table 2 *Digestion efficiencies of pasta, fish, meat investigated in this PhD thesis. *S/R indicates the sample-to-reagent tested; ** T indicated the temperature used in the experiments; ° indicated the mean calculated on three tests; § SD indicated the standard deviation;*

Sample	S/R*	T**	Time	Concentration	DE Mean°	SD§
		(°C)	(h)	(M)	(%)	
Fish	1:20	RT	48	5	84.0	5.2
	1:40	RT	48	5	82.8	8.5
	1:60	RT	48	5	79.3	0.6
Fish	1:20	50	48	5	97.4	0.4
	1:40	50	48	5	95.4	3.0
	1:60	50	48	5	93.2	4.2

In both cases, as the volume of reagent increases, other conditions being equal, the digestive efficiency is reduced (Figure 1). It clearly emerged that tests at 50°C gave a higher digestion efficiency.

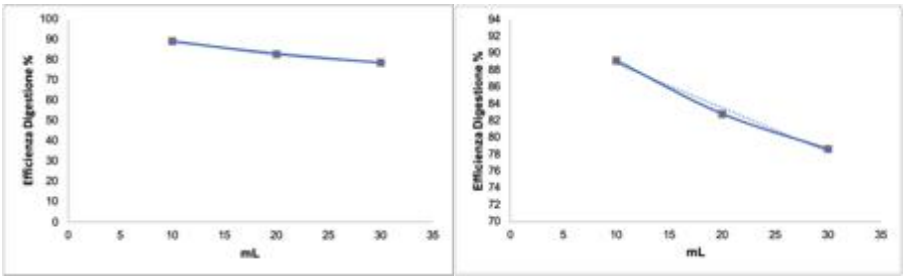

Figure 1 *Trend of digestive efficiency with varying volume (sample/reagent ratio) used for digestion. In order to clarify the potential dependence of digestive efficiency with the volume of reagent used, the graph is proposed with both full scale (left) and reduced scale (right).*

For meat and pasta, the type of digestion agents was investigated. KOH 5M showed the highest digestion efficiency, which was of 98.0±0.5%, whereas KOH 1M, Fenton's reagents and H_2O_2 (30%) showed digestion efficiencies of 88.4±2.2. 80.0±4.5 and 80.1±5.7, respectively. Regarding pasta, Fenton's reagent showed the highest efficiency 98.0±0.6. For other digestion agents, digestion efficiency was not calculated as the solutions were not able to be filtered.

5.2 Recovery rate

Recovery rate of microplastics were determined. For fish samples, recovery rate of microplastics was of 97±0.6%, by using KOH 5M (1:20) at 50°C for 48 h. For pasta samples, recovery rate was lower than fish one. It

was, in fact, of 78±1.7. This is potentially due to the fact that, even though digestion efficiency was high (98.0%), organic matter was still present on the filter (Figure 2, left). Red meat recovery rate was of 98±01%.

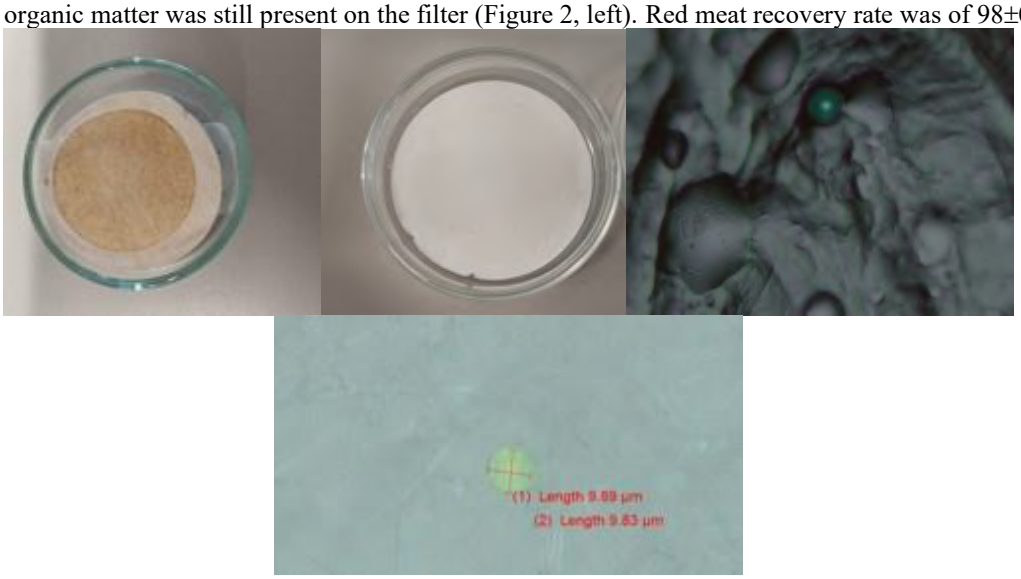

Figure 2 *Filters after digestion and filtration of pasta, using Fenton's Reagent (left) and meat sample, using KOH 5M (centre) and polystyrene fluorescent microbead before (on meat sample) and after digestion and filtration (right).*

5.3 Polymer solubilization into organic solvents

For polymers solubilization, results showed that PS was dissolved using by DCM or a mixture of DCM: toluene, as it was suggested by the theory. Even though chloroform was suggested by theory, it did not work during the experiments. Likewise, for PET, HFIP was the only organic solvent able to dissolve PET polymer. Other solvents emerged from the calculation based on the theory did not work. Finally, PE was tested with the mixture of solvents (n-hexane: toluene) suggested by the theory. However, solubilization did not occur.

5.4 Microplastic's quantification by Py-GC-MS

PS solubilised in DCM and PET in HFIP was spiked on fish samples in a final concentration of 100 ppb. Likewise, solutions at known and increasing concentrations were prepared and filtered for the calibration curves 'construction. After sample preparation steps, filter was cut and around 1 mm of the filter was placed into the liner and then placed in the pyrolysis cup for the analysis (Figure 3).

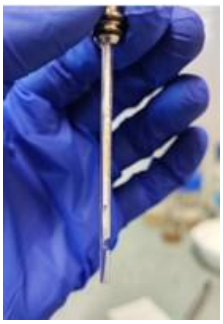

Figure 3 *Filter placed into the liner of the pyrolysis cup for the Py-GC-MS analysis.*

For the spectra obtained from the analyses, a strong background emerged. Basically, it can depend on the very important complexity of the food matrices.

Figure 4 *Chromatogram of a fish sample spiked with 100 ppb of PET. Benzophenone is the main pyrolysis product of PET, which can confirm the identification of the polymer in the sample.*

As for the calibration curves, results are still being validated. The main issue is the liner. It is, in fact, not able to host the entire surface of the filters, meaning that several analyses are necessary for obtaining reliable results.

6. Conclusions and Future Perspectives

The present PhD thesis would like to standardize the analytical methodologies currently applied in the microplastic's research field. Discrepancies are, in fact, still present in the field, and so, results obtained by different groups can be difficultly compared. Basically, in the scientific literature, the preparation of the samples is really poorly investigated. Most of papers stated the occurrence of microplastics, but several parameters such as quality control/quality assurance (QA/QC) as well as the digestion of the organic matter are under investigated. In this way, knowledges are required to fill the gap. In this thesis, results showed that even at high digestion efficiencies, the number of microplastics can be underestimated, as organic matter still present can totally hide them. At the same time, Py-GC-MS is a really promising techniques but, some investigation are still necessary to quantify microplastics reliably.

7. Nomenclature

W_i initial weight of digested material; W_a Weight of dry filter membrane after filtration; W_b Weight of dry filter membrane before filtration; N_a number of microplastics on the filter after extraction; N_b number of microplastics added to the sample before the extraction procedures; Ra is the solubility parameter; Ro is radius of the sphere of solubility; δ^d indicates the dispersion term of polymer and solvent; δ^p indicates the polar term of polymer and solvent; δ^h is the hydrogen bond term.

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Identification of Virulence Biomarkers in a *Listeria monocytogenes* ST7 Strain Through Immunoproteomic and Transcriptomic Analysis

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The main objective of this PhD project was to identify specific virulence biomarkers of a *Listeria monocytogenes* 1/2a strain, cultivated under different stress conditions, and to gain insights into the proteins implicated in its pathogenesis mechanisms.

Identificazione di biomarkers di virulenza in un ceppo di *Listeria monocytogenes* ST7 mediante analisi di immunoproteomica e trascrittomica

Il principale obiettivo del presente progetto di dottorato è stato quello di identificare specifici biomarker di virulenza in un ceppo di *L. monocytogenes* 1/2a, coltivato in diverse condizioni di stress e ottenere informazioni sulle proteine chiave coinvolte nei meccanismi di patogenesi.

Key words: *Listeria monocytogenes*; immunoproteomics; biomarkers; transcriptional profiling.

1. Introduction

The present PhD project aimed to identify specific virulence biomarkers of a *L. monocytogenes* ST7 strain grown at different stress environmental conditions. *L. monocytogenes* causes listeriosis, a severe foodborne infection in humans. The investigation of key proteins involving in virulence mechanisms of the pathogen is crucial to develop effective control strategies of public health surveillance. In order to achieve this objective, a comprehensive analysis of a *L. monocytogenes* 1/2a strain immunoproteome was conducted by mean of nLC-MS/MS technique combined with an immunoinformatic approach. Furthermore, a correlation between the immunoproteome and transcriptome was studied by the development of a bioinformatics pipeline, integrating data from both immunoproteomic and transcriptional profiling analyses. By combining different omics approaches, this project aimed to improve our understanding of *L. monocytogenes* pathogenicity and identify specific biomarkers which can help in early detection, risk assessment, and development of targeted interventions.

2. Materials and Methods

L. monocytogenes ST7 strain was cultivated at C1 (control): T 37°C, pH 7.0, NaCl 0.5%; C2: T 37°C, pH 5.5, NaCl 7%; C3: T 12°C, pH 7.0, NaCl 0.5%; C4: T 12°C, pH 5.5, NaCl 7% (D'Onofrio *et al*., 2021). Biological and technical triplicates were carried out for each experimental condition. The proteins were extracted, purified, and quantified. Protein extracts were resolved by SDS PAGE and Western blotting (WB) was carried out (D'Onofrio *et al*., 2022). The immunogenic proteins highlighted by WB were digested in-gel. The peptide extracts were analyzed by nLC-ESI-MS/MS, identified by Proteome Discoverer v1.4.1.14 against the database "uniprot_listeria_monocytogenes", and then analyzed by immunoinformatic pipeline (Paci *et al*., 2021). The proteins encoded only in C1 and C4 conditions were correlated with their respective transcripts, obtained by RNAseq analysis, using Cufflinks version 2.2.1 for transcript assembly and Tophat version 2.1.1 for transcripts abundance estimation, and differential expression analysis.

3. Results

In the present project an immunoproteomics approach combined with bioinformatics was applied to identify proteins showing differential expression in *L. monocytogenes* ST7 strain under different stress conditions and therefore relevant for the presence of the strain in meat products. By detecting antigen-antibody immunocomplexes (Ag-Ab ICs), potential immunogenic proteins specific to each stress condition have been predicted by different bioinformatic tools.

Through a comprehensive analysis using nLC-ESI-MS/MS-based proteome identification, a total of 226 proteins were identified. Among them, 58 proteins (28.3%) were classified as potential antigens, of which 6 membrane proteins were detected as unique in the 3 experimental stress conditions (C2, C3 and C4).
Based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, membrane proteins were involved in the functional categories as listed below.

A1) General stress response: Q8Y3Z9 (*lmo2679*), O32823 (*trxB*), Q8YAJ3 (*lmo0132*).
A2) Cell morphology and motility: Q8Y942 (*lmo0699*), Q8Y919 (*lmo0723*).
A3) Carbohydrate transport and metabolism and energy production: Q8Y864 (PdhB).

3.1 Immunoblotting

Immunoblotting analysis was conducted using a *L. monocytogenes* positive ovine serum (Fig. 1). Specific Ag-Ab ICs were detected at 50 kDa and 40 kDa under all growth conditions. Furthermore, Ag-Ab ICs were observed at 110 and 75 kDa in C2 and C3, respectively. Then, specific Ag-Ab ICs were identified at 80 kDa in C3 and C4, and at 60 kDa in C1 and C4.

3.2 Bioinformatic

A total of 226 proteins were identified using nLC-ESI-MS/MS. To predict the subcellular localization of selected proteins, different tools were utilized, including LipoP 1.0 Server, TMHMM Server version 2.0, SignalP 4.1 Server, PSORTb version 3.0.2, and CELLO version 2.5. Among the identified proteins, 76 were non-cytosolic. These proteins were further analyzed using NetSurfP version 1.1 and BepiPred version 1.0 Server in order to assess their localization and predict epitopes exposed to solvent. Fifty-eight proteins were classified as potential antigens using Virulent Pred, Vaxign tool, and VaxiJen Server. Among them, 30 proteins were exclusively present in C1, while 6 proteins were identified in C2, C3, and C4 (Fig. 2).
A specific histidine kinase with a molecular weight (MW) of 100 kDa was found exclusively in C2. Only in C3, the flagellar motor switch protein (FliM) with a MW of 38 kDa was identified, together with lmo0723 (MW 66 kDa) encoded by the gene *lmo0723*.
In C4, the proteins identified were PdhB (MW 35 kDa), which bears similarity to pyruvate dehydrogenase (E1 b-subunit), thioredoxin reductase (MW 34 kDa), and lmo0132 (MW 55 kDa) involving in DNA repair and maintenance (Table 1) (Wang *et al*., 2016). Moreover, in C4 the expressed proteins were associated with a higher degree of stress, which could lead to cell damage and cell death. One of the identified proteins was the pyruvate dehydrogenase E1 beta-subunit (PdhB), encoded by the *lmo1053* gene. PdhB is part of the pyruvate dehydrogenase enzyme complex (PDH), which played a role in carbohydrate metabolism. Another expressed protein in C4 was thioredoxin reductase, encoded by *trxB*. This protein is considered part of the oxidative stress response and is regulated by the peroxide operon PerR. Q8YAJ3, encoded by *lmo0132* was identified in C4 condition.

3.3 Transcriptomic

The integrated analysis of immunoproteomics and transcriptomics data provided valuable insights into the activation of stress response genes in C4. Among them, gene *lmo0613* encoding for lmo0613 proteins-oxidoreductase, exhibited significant upregulation. The oxidoreductases played a crucial role in increasing resistance to oxidative and antibiotic stress in bacteria (Pleitner *et al*., 2014). The activation of lmo0613 suggested that *L. monocytogenes* employed this pathway to cope with the challenging environmental conditions in C4. Another gene, *lmo1525*, encoding for the recombination protein RecS, also displayed pronounced upregulation in C4. Recombination proteins, such as RecS, are known to contribute to various cellular processes, including cold tolerance and DNA repair and recombination. The upregulation of lmo1525 indicated that *L. monocytogenes* activates DNA repair mechanisms and recombination pathways in response to the combined stressors in C4.

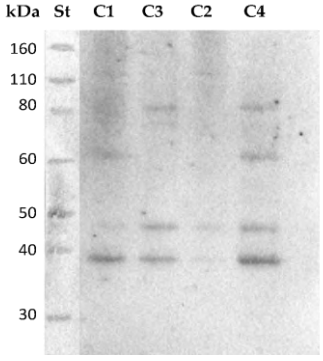

Figure 1 *Immunoblotting of L. monocytogenes ST7 strain, cultivated at four different environmental conditions (St: Standard; C1: 37°C, pH 7.0, NaCl 0.5%; C2: 37°C, pH 5.5, NaCl 7%; C3: 12°C, pH 7.0, NaCl 0.5%; C4: 12°C, pH 5.5, NaCl 7%).*

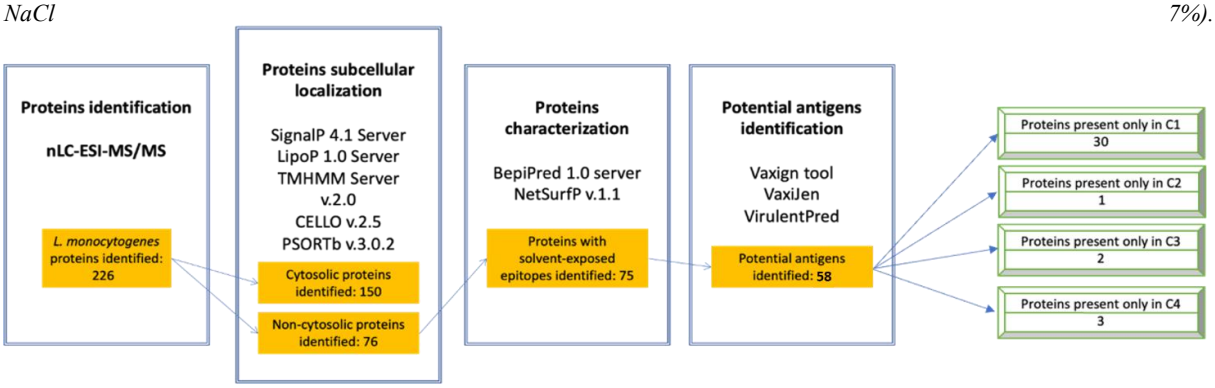

Figure 2 *Identification of immunogenic proteins of L. monocytogenes ST7 cultivated at four different combinations of T, pH and NaCl %: bioinformatic workflow (St: Standard; C1: 37°C, pH 7.0, NaCl 0.5%; C2: 37°C, pH 5.5, NaCl 7%; C3: 12°C, pH 7.0, NaCl 0.5%; C4: 12°C, pH 5.5, NaCl 7%).*

Table 1 *List of identified L. monocytogenes ST7 immunogenic proteins (St: Standard; C1: 37°C, pH 7.0, NaCl 0.5%; C2: 37°C, pH 5.5, NaCl 7%; C3: 12°C, pH 7.0, NaCl 0.5%; C4: 12°C, pH 5.5, NaCl 7%).*

Gene	Protein		Function	Localization	MW (kDa)	Condition
lmo2679	**Q8Y3Z9**	Histidine kinase	Adaptation to high-osmolarity conditions by regulating the expression of a high-affinity potassium uptake system encoded by the *kdpABC* genes	Membrane	100	C2
lmo0699	**Q8Y942**	Flagellar motor switch protein - FliM	Part of the switch complex that is involved in switching the direction of the flagella rotation	Membrane	38	C3
lmo0723	**Q8Y919**	lmo0723	Methyl-accepting chemotaxis protein	Membrane	66	C3
lmo1053	**Q8Y864**	PdhB protein	Highly like pyruvate dehydrogenase (E1 beta-subunit)	Membrane	35	C4
trxB	**O32823**	Thioredoxin reductase	Protection against oxidative stress by regulating the expression of thioredoxins	Membrane	34	C4
lmo0132	**Q8YAJ3**	lmo0132	DNA repair and mantainance	Membrane	55	C4

4. Discussion

Listeriosis is recognized as the fifth most frequently reported foodborne disease among humans, and its notification is mandatory in the European Union (EFSA and ECDC, 2021). Consequently, the prevention and control of *L. monocytogenes* continue to be of greatest importance to ensure food safety worldwide. In recent times, different omics approaches, including proteomics, genomics, and transcriptomics, have emerged as valuable tools for studying the biodiversity of *L. monocytogenes* strains. In particular, immunoproteomics and transcriptomics provide appreciated insights into the behavior of the pathogen under stress conditions.

In response to stressors, *L. monocytogenes* can undergo changes in cell morphology, motility, and metabolism to enhance their growth and survival. The present study revealed the presence of histidine kinase (Q8Y3Z9) encoding by the *lmo2679* gene when the microorganism is exposed under mild acid and salt stress conditions at 37°C (C2). Histidine kinases are transmembrane proteins that play crucial roles in two-component regulatory signaling systems that induce the transcription of genes encoding membrane desaturases, thereby enhancing membrane fluidity. The expression of the histidine kinase encoded by *lmo2679* could be a response to the combined stresses of acidity and salt. In C3, where the only stressor was low temperature, only 2 expressed proteins involved in cell motility were identified.

Previous studies have demonstrated that *L. monocytogenes* can modulate flagellar motility in response to

temperature, salt stress, and pH (Horlbog *et al*., 2019). The genes associated with cell motility in *L. monocytogenes* were downregulated under stress conditions. This regulation is crucial because flagella consume energy and could trigger the immune response. In *L. monocytogenes*, the flagellar motor-switch protein FliM, encoded by *lmo0699*, is essential for motility and involved in the secretion of other flagellar-related proteins. Most strains exhibit motility below 25°C, so it is not surprising to find the expression of FliM at 12°C. However, in the presence of acid and salt stress (C4) at the same temperature, FliM (lmo0699) was not detected. This finding suggests the impact of combined stress factors on the proteome. Interestingly, another protein Q8Y919, encoded by *lmo0723*, was identified in C3. Q8Y919 is potentially involved in chemotaxis and motility. In C4, the expressed proteins were associated with a higher degree of stress, which could lead to cell damage and cell death. PdhB, encoded by the *lmo1053*, plays a role in carbohydrate metabolism. TrxB, encoded by *trxB*, is considered part of the oxidative stress response, and is regulated by the peroxide operon PerR. TrxB is upregulated in response to harsh environmental conditions, such as after 48 h of desiccation on stainless steel surfaces and under acid shock at pH 3.0 with HCl in highly acid-tolerant field strains (Horlbog *et al*., 2019). This protein is an enzyme involved in the purine biosynthesis pathway called inosine 5-monophosphate dehydrogenase. It catalyzes the synthesis of xanthosine monophosphate through the NAD+-dependent oxidation of inosine monophosphate. The upregulation of genes, involved in severe stress response and DNA repair, suggested that the cultivation conditions used in C4 induced a response to extreme environmental conditions.
The correlation between immunoproteomics and transcriptomics demonstrated the activation of numerous stress response genes under the most severe stress conditions, such as *lmo0613* and *lmo1525*, involved in cold tolerance and antibiotic resistance, respectively. This observation highlighted a robust and coordinated cellular response by *L. monocytogenes* to the combined stressors present in C4.

7. Conclusions and Future Perspectives

In conclusion, the present study focused on understanding the proteome' changes of *L. monocytogenes* when cultivated under sub-optimal temperature along with mild acid and salt stress conditions commonly found in meat products. Despite extensive research into the stress response of *L. monocytogenes*, this pathogen continues to pose a significant threat in food production environments, raising concerns for consumer safety. By studying the functions of different expressed proteins and relating them to their transcripts, this research has contributed to understanding their potential roles in the emergence or reemergence of listeriosis outbreaks. The identification of effector proteins secreted under real-time conditions has provided valuable information that can help in the development of new strategies for the treatment and prevention of listeriosis.

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Biocatalytic modification of monoterpenes using waste-derived yeast cells

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This PhD thesis focused on the development of a sustainable process for the reduction of perillaldehyde to perillyl alcohol (POH) by employing yeast cells immobilized into calcium alginate beads (entrapment technology). After optimizing the reaction conditions in small-scale batch reactions, process was scaled in a rotating bed reactor (SpinChem) reaching 90% of molar conversion. The biomass used as biocatalyst was produced starting from waste material and using seawater instead of the fresh one. By harnessing the potential of immobilized yeast cells in a rotating reactor and employing environmentally friendly resources, this study exemplified a sustainable biocatalytic approach with the potential to be expanded to other natural molecules.

Processo sostenibile per la modifica di terpenoidi utilizzando lieviti immobilizzati

Questa tesi di dottorato si è concentrata sullo sviluppo di un processo sostenibile per la produzione di POH utilizzando cellule intere di lievito immobilizzate via intrappolamento in idrosfere di alginato. Dopo aver ottimizzato le condizioni in batch (1-2 mL), la reazione è stata scalata in un reattore a letto rotante (SpinChem) raggiungendo il 90% di conversione molare. La biomassa utilizzata come biocatalizzatore è stata prodotta partendo da materiale di scarto e utilizzando acqua di mare anziché acqua distillata. Sfruttando il potenziale di cellule immobilizzate in reattore e impiegando materiale eco-sostenibile, questo studio è un esempio di approccio biocatalitico sostenibile con il potenziale di essere esteso a diverse molecole di origine naturale.

Key words: Biocatalysis, rotating bed reactor, monoterpenoids, circular economy, enzyme immobilization.

1. Introduction

This oral communication reports the main results of the following four activities directed to:
A1) Screening of yeast collections to identify strains able to modify monoterpenes;
A2) Optimizing the biotransformation conditions in small scale batch reactions;
A3) Scaling-up the process in a rotating bed reactor (SpinChem);
A4) Enhancing the sustainability of the whole process by utilizing of waste materials and seawater.

2. Whole cells biocatalysis

Biocatalysis involves the use of biological systems (whole cells or enzymes) to catalyse chemical reactions. This approach has gained attraction in various industries, including pharmaceuticals, chemicals, cosmetics, agriculture, and food companies, due to its potential for sustainable, energy-efficient, and environmentally friendly production processes. Indeed, enzymes operate under mild reaction conditions, such as moderate temperatures, water media (neutral pH), and atmospheric pressure, preserving the biocatalyst activity and avoiding the massive use of solvents. Enzymes can be utilized as isolated proteins, cellular extracts, or within whole cells, either free or immobilized. Isolated enzymes require a long and usually expensive process for their purification, and their recovery and reuse are difficult. On the contrary, whole cells do not require purification steps and can be easily recovered from the culture medium, thus reducing production time and costs (Schrewe *et al*., 2012). In addition, the use of whole cells is usually preferred for reactions involving cofactors as they can synthesize and recycle cofactors themselves (Haque *et al.*, 2019; Filippucci *et al.*, 2020). However, cells present intrinsic metabolic pathways, so competitive secondary reactions due to the presence of other enzymes may occur with the risk of reduced final yields as well as the formation of undesired by-products.
To enhance the usability of whole cells, immobilization techniques can be employed, such as encapsulating or immobilizing them within hydrogel beads that serve as carriers for the biocatalysts. These hydrogel beads provide a protective environment for the cells, allowing them to retain their activity and stability during the reaction process. The immobilization also facilitates the isolation of the product and the separation of the catalyst from the reaction mixture, simplifying downstream processing for industrial scale-up (Rodrigues and de Carvalho, 2022).
Moreover, the use of immobilized catalysts in the SpinChem reactor system, which efficiently facilitates liquid percolation through packed particle bed within the stirring element, presents additional benefits (Mallin *et al*., 2013).

Overall, biocatalysis offers established and significant advantages and continues to be explored and optimized for various applications, driving the shift towards sustainable and environmentally conscious production processes.

3. Modification of monoterpenes

Terpenoids are a diverse group of natural compounds primarily derived from plants, that find applications in various industries such as food, pharmaceuticals, and cosmetic ones. The use of yeast whole cells for the biotransformation of certain monoterpenoids, including carvone, geraniol and limonene, into highly valuable flavoring derivatives has been studied due to their economic potential in the food and beverage, perfume, and soap industry (Goretti *et al*., 2011; van Beilen *et al.*, 2005). Perillaldehyde is a terpenoid found in various plants and essential oils, with perilla herb being the most abundant source. It is commonly used as a food additive for flavoring and in perfumery to provide a spicy aroma. However, in 2015, the European Food Safety Authority (EFSA) conducted an evaluation and determined that perillaldehyde demonstrated genotoxic potential in vivo, raising safety concerns as a flavoring substance (Hobbs *et al.*, 2016; Erhunmwunsee *et al.*, 2021). Subsequently, the European Commission announced its intention to remove perillaldehyde from the EU list of flavorings (http://eur-lex.europa.eu/eli/reg/2015/1760/oj).
Taking into consideration the concerns raised by the use of perillaldehyde as food ingredient, this compound can be used to produce the correspondent natural derivative, perillyl alcohol (POH), which many studies investigated as a possible therapeutic agent in the prevention and treatment of cancer (Shojaei *et al*., 2014). Indeed, POH showed the ability to inhibit different type of tumors at different stages, such as skin, liver, breast, glioma, lung, colon and gastric cancers in animal models (Chen *et al*., 2021). However, this natural compound is present only in low quantities in a few plant oils, so an alternative source of POH is becoming necessary (Chen *et al*., 2021). Although POH can be produced via classical organic synthesis, for example, through the hydrogenation of α,β-unsaturated aldehydes, in recent years there has been a significant preference towards biotransformation processes carried out by using microbial cells as biocatalysts for the production of fine chemicals (Forti *et al.*, 2015). Accordingly, molecules obtained by such bioprocesses can be labeled as 'natural' and GRAS ('Generally Regarded As Safe'), thus increasing their market value (Contente et al., 2020).

5. Materials and Methods

The strain *Candida viswanathii* Bio1 (UBOCC-A-208001) was grown in baffled flasks or in 2L-bioreactor (Applikon), using YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) or molasses (1:2) pre-treated with H_2SO_4 1.5% (v/v) mixed with filtered and sterilized seawater and yeast extract (1 g/L). The fed-batch process was set as follow: 28 °C, pH maintained at 6, oxygen over 40%. For the biocatalyst immobilization, 200 OD of cells were mixed 1:1 with a 5% w/v solution of sodium alginate and dropped into a 0.2 M $CaCl_2$ solution thought a P200 tip to form beads of size 3 mm each. Biotransformations were performed in 5 mL glass vials, at room temperature (23-25°C), in continuous stirring at 150 rpm. Biotransformation products and substrates, after extraction with EtOAc 1:1, were analyzed through TLC (hexane/EtOAc 7:3, revealed by vanillin staining), ^{1}H-NMR and gas chromatography (GC).

5. Results and Discussion

5.1 Screening for monoterpenes reduction

A total of fourteen yeast wild-type strains, listed in Table 1, were screened for their ability to modify monoterpenes through biotransformation for the achievement of interesting APIs (Active Pharmaceutical Ingredients). For the first screening, (R)- and (S)-carvone were added at the concentration of 1 g/L after 24 hours of cultivation in rich medium (YPD). The cultural broth was collected after another 24 hours and analyzed by TLC. Interesting results were shown by three yeast strains: CEN-PK and the non-conventional yeast strains Bio1 and CCAT2. These three strains were tested also on other terpenes (Fig. 1): (R)-carvone (a), (S)-carvone (b), (S)-perillaldehyde (c), thymoquinone (d), (R)-pulegone (e), verbenone (f), and β-ionone (g). Notability, Bio1 was the only strain to show positive results for all substrates (except for verbenone and β-ionone).

Species	Strain	Source
Saccharomyces cerevisiae	CEN-PK	Laboratory strain
Dekkera bruxellensis	Y908	Grape must
	Y911	Equipment in beer brewery
	Y871	Sour wine
	Y870	Sour wine
	Y906	Tea-beer
Kluyveromyces lactis	CBS2359	Creamery

Trichosporon oleaginosus	CCAT2	Dairy plant
Debaryomyces hansenii	Mo40	Deep-sea hydrothermal vents
Candida viswanathii	Bio1	Deep-sea hydrothermal vents
Lypomyces lipofer	LLDP5	Soil
Rhodosporidium paludigenum	CBS6566	Juncus roemerianus (marsh)
Rhodosporidium azoricum	RGRDP3	Soil
Rhodotorula glutinis	RGNR2	Air

Table 1 *Yeast strains screened for monoterpenes biotransformation.*

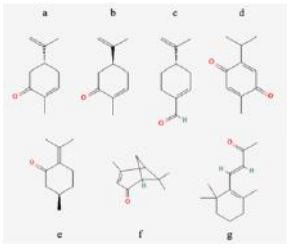

Figure 1 *Table of terpenes tested on Bio1 and CCAT2 strains.*

5.2 Cell immobilization and optimization of batch biotransformation

Based on the screening results, we decided to focus on the reduction of (S)-perillaldehyde into POH performed by Bio1 strain as a model reaction. The putative enzyme involved in this conversion is a carbonyl reductase which catalyzes the reduction of the carbonyl group by using cofactors (Fig. 2). To obtain a more stable and easier to use biocatalyst, Bio1 cells were immobilized into Ca-alginate hydrogel beads. The media for the biotransformation was composed of physiological solution (NaCl 0.9%) supplemented by glucose (40 g/L) to enhance the cofactor recycling system (Filippucci *et al.*, 2020; Goretti *et al.*, 2009). Working on small volumes (1 or 2 mL), different concentrations of alginate beads were tested with increasing concentrations of substrate (Table 2, 3). The best result (89% molar conversion) was archived by using 1 g/mL of immobilized biocatalyst with 4 mM of perillaldehyde in 6-hour reaction time (Table 4).

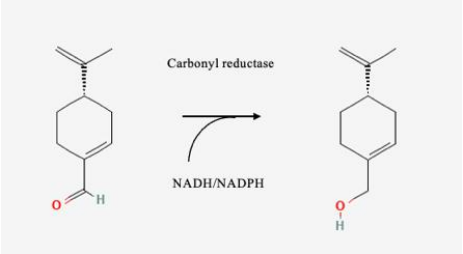

Figure 2 *Reduction of (S)-perillaldehyde into (S)-perillyl-alcohol.*

Table 2 *Molar conversion obtained using different amount of immobilized biocatalysts (g of alginates/mL).*

Immobilized catalyst (g/mL)	**POH** (mM)	**Molar conversion** (%)
0.1	2.83 ± 0.22	35
0.5	3.50 ± 0.28	44
1	4.18 ± 0.16	53

Table 3 *Molar conversion obtained using 1 g/mL of immobilized biocatalysts with increasing substrate concentrations.*

Perillaldehyde (mM)	**POH** (mM)	**Molar conversion** (%)
4	3.43 ± 0.26	90
7.5	4.18 ± 0.22	54
14	5.34 ± 0.14	37

Table 4 *Molar conversion obtained using 1g of alginates and 4 mM of substrate.*

Time (h)	**Molar conversion** (%)
2	76
4	68
6	89
24	85

5.3 Attempt to scale the process through a flow system

As a first attempt to scale up the process, we tried to transfer the reaction into a flow system. Specifically, a 12 cm x 0.4 cm glass column (4 mL volume) was packed with 4 g of alginate beads containing Bio1 cells. The stream containing the physiological solution with the substrate was set at 0.3 mL/min (residence time: 15 min). Unfortunately, this system did not work as expected because perillaldehyde adhered to the plastic tubing of the system, resulting in very low conversion as the substrate did not come into contact with the biocatalyst.

5.4 Scale-up in a rotating bed reactor (SpinChem reactor)

The SpinChem reactor is a type of rotating bed reactor (RBR) that exploits centrifugal acceleration to enhance mixing and mass transfer in the system (Fig. 3). Additionally, this reactor facilitates easy scalability, making it suitable for both laboratory-scale and industrial-scale applications. The SpinChem reactor is ideal to be used with cells immobilized in hydrogel beads as catalysts (Fig. 4).

Using this system, more than 90% of molar conversion was reached after 8 hours in a working volume of 200 mL (Table 5). More interestingly, this result was obtained using 10 times less biocatalyst compared to small scale reactions (0.1 g/mL compared to 1 g/mL). The efficient design of the system allowed for effective interaction between the immobilized cells and the reaction medium, ensuring uniform distribution of reactants and enhancing reaction kinetics and overall performance.

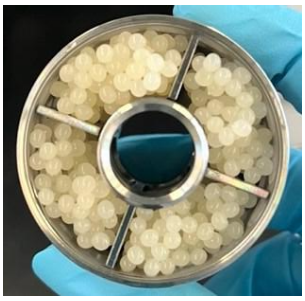

Figure 3 *SpinChem reactor system.*
Figure 4 *The internal cage of a SpinChem fulfilled with the biocatalyst immobilized into Ca-alginate hydrogel beads.*

Table 5 (*S)-perillyl-alcohol production in the SpinChem reactor.*

Time (h)	(S)-perillaldehyde (mM)	(S)-perillyl-alcohol (mM)	(S)-perillyl-alcohol (mg/L)	Molar conversion (%)
0	4.16 ± 0.08	-	-	-
1	3.51 ± 0.08	0.98 ± 0.05	148	24
2	3.04 ± 0.07	1.85 ± 0.17	281	45
3	2.65 ± 0.01	2.52 ± 0.14	384	62
4	2.33 ± 0.02	3.33 ± 0.35	506	81
6	1.71 ± 0.01	3.07 ± 0.01	467	75
7	1.36 ± 0.17	3.41 ± 0.01	518	83
8	0.29 ± 0.01	4.02 ± 0.03	607	91

5.5 Sustainability focus: use of waste materials and seawater

To adhere to circular economy principles and prioritize sustainable resource management, we evaluated the growth ability of Bio1 strain on a waste-derived medium based on molasses, a by-product of sugar beet refining, instead of the expensive synthetic medium. In addition, considering that Bio1 is a marine yeast strain, we chose to employ seawater instead of distilled water, as it contains essential minerals and reduces the risk of contamination.

Unfortunately, we found that Bio1 strain was not very efficient at metabolizing sucrose, so an acidic pre-treatment of molasses was needed to promote the hydrolysis of sucrose into glucose and fructose. In addition, a small amount of yeast extract (1 g/L, 10 times less than the synthetic medium) was added to stimulate the growth. With this strategy, we were able to produce more than 35 g/L (dry weight) of biocatalyst in 70 hours of process in 2L bioreactor.

To further enhance the sustainability of the overall process, we also decided to replace the physiological solution used as biotransformation medium with seawater, obtaining comparable results in terms of both molar conversion and product titers (data not shown).

6. Conclusions and Future Perspectives

This study serves as a proof of concept to demonstrate the viability and potential applicability of the proposed approach for biocatalytic processes, paving the way for further exploration and utilization in related research areas. The results show the high potential of non-conventional yeasts as biocatalysts for the biotransformation of cheap and largely available monoterpenes (*i.e.,* those present in the screening but not limited to) to valuable derivatives, that have the potential to be explored as potential active pharmaceutical ingredients (APIs) and claimed as 'natural' products, following the European and US regulation. Furthermore, the incorporation of waste materials into the process emphasizes the commitment to sustainability and aligns with the principles of a circular economy. By utilizing molasses and seawater as feedstocks and incorporating them into the production cycle, the overall process becomes more environmentally friendly and resource efficient.

7. Nomenclature

POH, peryllil-alcohol; g/L, Grams per Liter; YPD, Yeast Extract–Peptone–Dextrose broth; APIs, potential active pharmaceutical ingredients; EtOAc, ethyl acetate; TLC, thin layer chromatography; RBR, rotating bed reactor.

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Development of an electrochemical sensor to detect micro and nanoplastics in environmental and agri-food samples

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This PhD project dealt with the development of a sensor for micro and nanoplastics detection. Electrolyte-gated Field-effect-transistor (EG-FET) was the sensor of choice; carbon nanotubes (CNTs) were employed as the semiconductor material (i.e., active layer) of the sensor. The possible interaction between CNTs and nanoplastics was exploited; in fact, in EG-FETs, rearrangements of the CNTs network (upon interaction with an analyte) lead to changes in the measured current. Morphological, electrical and chemical characterizations were performed on the devices to confirm our hypothesis.

Sviluppo di un sensore elettrochimico per il rilevamento di micro e nanoplastiche in campioni ambientali

Lo scopo del progetto di dottorato è di sviluppare un sensore per la rilevazione di micro-nanoplastiche. Un sensore basato su un transistore ad effetto di campo è stato scelto; i nanotubi di carbonio (CNTs) sono stati usati come materiale semiconduttivo. La possibile interazione tra CNTs e nanoplastiche è stata studiata e sfruttata per il sensore; infatti nei transistori, perturbazioni e quindi una possibile riorganizzazione nel network di CNTs (dovute alla presenza di un analita), risultano in un cambiamento della corrente misurata. Per confermare l'ipotesi, caratterizzazioni morfologiche, elettriche e chimiche sono state effettuate sui sensori sviluppati.

Key words: EG-FET; nanoplastics; carbon nanotubes; electrochemical sensors; contaminants.

1 Introduction

The goal of the PhD project was to develop a sensor to detect micro and nanoplastics (NPs); the sensor of choice was the Electrolyte-gated Field-effect-transistor (EG-FET), which is nowadays started to being used for studies related to environmental monitoring and protection (Elli et al, 2022). Here, we are presenting an EG-FET sensor for NPs detection, overcoming the limitations of standard spectroscopic techniques, such as high cost and longtime processing. In accordance with the PhD project, here are reported the practical activities carried out and developed during the three years of PhD:

A. Optimization of the fabrication process of the EG-FET based sensor (such as photolithography parameters and carbon nanotubes (CNTs) spraying parameters);
B. Stability test with only deionized water (DI-water) and artificial seawater, on the fabricated EG-FETs;
C. Test with different polystyrene (PS) NPs concentrations to assess the device response. Both using DI-water and artificial seawater as electrolyte solution;
D. Test with polyethylene terephthalate (PET) NPs, to compare two different plastic materials;
E. Studies on the interaction between PS NPs and other contaminants, such as metal ions and pesticides.

2 Materials and Methods

2.1 Device fabrication

EG-FET devices were fabricated on glass substrate, by employing negative photolithography, to pattern the desired design. This was followed by thermal evaporation of Chromium (Cr, 10nm) and Gold (Au, 50nm) and lift-off step in acetone, as described in Shkodra et al. (2022). Semiconducting CNTs were spray coated on top of the interdigitated contacts followed by 1 hour of treatment in nitric acid (2.9 M) to remove the solvent.

2.2 Device stability

The electrical response of the devices to water was first tested (at probe station - Keysight B1500A semiconductor device parameter analyzer); a plastic microchamber was put on top of the interdigitated contacts and the gate electrode, as a way of incapsulating the liquid solution. The stability of the devices in DI-water was tested, and transfer curves (drain to source current (I_{DS}) measured, with fixed V_{DS} of -100 mV and V_{GS} swept from 500 to -500 mV) were taken every 5 minutes for 2 hours. Output curves were taken after 7 minutes of the test, fixed V_{GS} (5 different values, from 0 to -800 mV with a step of -200 mV), with a sweep in V_{DS} from 0 to -500 mV. Subsequent four additions of more volume of DI-water were done, and transfer curves were taken every 5 minutes with the same parameters. To compare different devices, I_DON (at V_{GS} -800 mV) and I_DOFF (at V_{GS} 400 mV) (of the transfer curves) were normalized with the following equations:

$$Normalized\ I_DON\ (\%)_x = \frac{I_DON_X}{I_DON_{5min}} * 100 \qquad (1)$$

$$Normalized\ I_DOFF\ (\%)_x = \frac{I_DOFF_X}{I_DOFF_{5min}} * 100 \qquad (2)$$

The same test was performed with artificial seawater (Utex, Bio Labs 218, UT Austin; Austin, Texas, USA), which has a pH of 8.19 and conductivity of 31.8 mS/cm. The only difference was that the extra additions of water were only two and not four like for DI-water.

2.3 Nanoplastics tests

PS test was performed to study the interaction of PS NPs with CNTs. PS nanobeads with 100 nm diameter (Alpha Nanotech Inc., Canada), were used. First, 92 minutes of stabilization in DI-water was performed, where transfer curves were taken every 5 minutes with the same parameters as in 2b. Then PS solution was added in the microchamber, every 25 minutes (90 µl every time), while transfer curves were taken every 5 minutes (and output curves after 7 minutes) with the same parameters as in 2b. Six concentrations of PS were tested: 0.05, 0.1, 0.25, 0.5, 1 and 2 mg/ml. For the stabilization part, I_{DS} was normalized using equations 1 and 2; for the PS solution part, these equations were instead used:

$$Normalized\ I_DON\ (\%)_x = \frac{I_DON_X}{I_DON_{92min}} * 100 \qquad (3)$$

$$Normalized\ I_DOFF\ (\%)_x = \frac{I_DOFF_X}{I_DOFF_{5min}} * 100 \qquad (4)$$

ON/OFF ratios were obtained by simply dividing I_DON (at V_{GS} -800 mV) by I_DOFF (at V_{GS} 400 mV).

The same test was performed with artificial seawater instead of DI-water as electrolyte solution; for this test, artificial seawater was always used in a 1:5 dilution with DI-water. A similar test was conducted also with PET NPs. In this case, PET NPs were fabricated in IIT (Istituto Italiano di Tecnologia) using a method called laser ablation, as described in Magrì et al. (2018). PET NPs are heterogenous in size and shape, so to compare with PS, the concentrations were converted to particles/ml. Only the three lower concentrations of PS were used for the test with PET, because of the low availability of the material.

2.4 Morphological and chemical characterization

Atomic force microscope (Nanosurf CoreAFM, Nanosurf AG, Switzerland) images of the interdigitated contacts were taken before and after NPs test (NPs solution was removed and devices were rinsed with DI-water). Scanning electron microscope (SEM) was also used to take images on interdigitated contacts before and after PS NPs test. X-ray photoelectron spectroscopy (XPS) was performed using an electron spectrometer (Lab2, Specs, Berlin, Germany) equipped with a monochromatic X-ray source (set at 1486 eV) and with a hemispherical energy analyzer (Phoibos, HSA3500, also from Specs). The samples were analyzed in the interdigitated contacts; spectra of these samples were acquired: gold + CNTs, gold + PS NPs (drop-casted), gold + CNTs + PS NPs (after PS NPs test).

2.5 PS interaction with contaminants

The ability of PS to adsorb different environmental contaminants on its surface, and create PS-contaminant complexes was studied. The contaminants of choice were mercury ions (Hg^{2+}) and glyphosate (a pesticide). The experimental setup was the same for both contaminants: 1 ml of PS suspension in Milli-Q water (300 µg/ml) was pelleted by centrifugation at 21460 xg for 30 min at 25°C. The supernatant was removed (but collected for further analysis) and substituted with the same volume (1 ml) of Milli-Q water solutions of glyphosate (40 µg/ml) or of Hg^{2+} (25 µg/ml) (all chemicals supplied by Sigma-Aldrich). The suspensions were then incubated for 48 h under shaking (around 450 rpm) at room temperature, and then transferred in a new centrifuge tube and pelleted (21460 xg, 30 min, 25°C). The pellets were then resuspended in 1 ml of Milli-Q water to wash them; this procedure was repeated four times.

2.6 PS-contaminants complexes characterization

UV-Vis spectrophotometer (Varian Cary 6000i) was used to measure the concentration of PS and glyphosate in the supernatant collected after each centrifugation step. For both, first a calibration curve was done; PS absorbance peak was at 203 nm, glyphosate was at 189 nm. Inductively coupled plasma (ICP) analysis were performed to measure the concentration of Hg^{2+}. The supernatant collected after centrifugation, in each washing step of PS-Hg adsorption tests, was tested. Samples preparation for ICP analysis was as follows: liquid sample was added to a flask (~800 µl), then aqua regia solution (3/4 HNO_3 and 1/4 HCl) was added (10% of the flask total volume, the rest Milli-Q water). The solution was then filtered (filter with 200 µm mesh). Binding capacity of PS and contaminant was measured using the following equation, where Hg^{2+}_{bound} was obtained by subtracting the amount of Hg^{2+} recovered in the supernatants to the starting amount (same for PS final concentration):

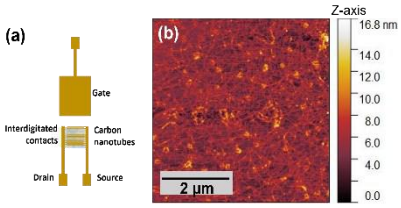

***Figure 1**: a) Schematic of the EG-FET, with gold electrodes (gate, source and drain) in a co-planar configuration. b) AFM image of CNTs network on the interdigitated contacts (glass part).*

$$Binding\ capacity = {}^{[Hg^{2+}_{bound}]}/_{[PS]} * 100 \qquad (5)$$

XPS analysis was then performed on the PS-Hg complexes, to confirm their chemical interaction.

3 Results and Discussion

3.1 EG-FETs characterization and stability

Glass was used as substrate for the devices, the main advantages of this material are the transparency, that could be exploited for optical studies, and the ability to withstand high operating temperatures. EG-FET was the chosen configuration of the device, and a schematic of the design is shown in figure 1a; the three electrodes are in a co-planar configuration. After CNTs spraying, AFM images were taken of the devices; in figure 1c, the network of CNTs is visible; the image was taken on glass part of the interdigitated contacts. It was observed that upon addition of electrolyte (liquid) solution, the EG-FETs were not stable (i.e., I_{DS} measured decreased with time, especially I_DON); a study on their stability was performed, and at first, stability in DI-water was studied. It was noticed that with DI-water, the decrease was of 20% after 22 minutes, almost 1% per minute; then after 70 minutes I_DON presented a good stability, with a small linear decrease of less than 1% every 5 minutes, as presented in figure 2a. Addition of more volume of DI-water did not influence I_DON, which remained stable (total decrease of less than 5%), meaning that a change in volume does not drastically affect the devices. The stability of the devices was studied also in a more complex medium, artificial seawater, which contains salts. In figure 2b, the normalized response of 4 devices is shown; I_DON was stable after about 60 minutes of test (total decrease of 18%, with a decrease of less than 1% every 5 minutes after 60 minutes). A difference with DI-water was the I_DOFF, in fact with artificial seawater it increased over time (of 15% at the end of the test), while for DI-water it decreased with time (of almost 70%). Two additions of artificial seawater were done, also in this case I_DON and I_DOFF were not affected by the change in volume.

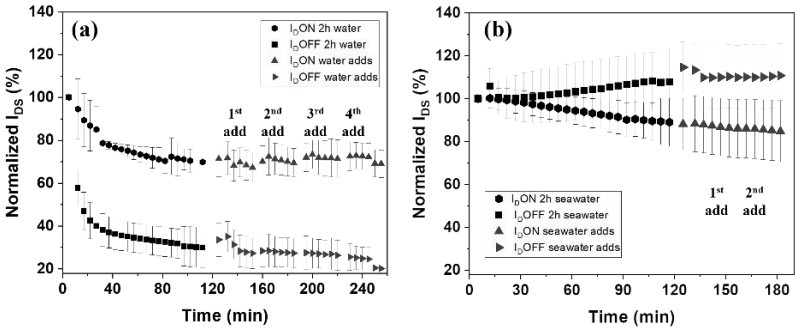

***Figure 2:** a) DI-water stability test. Normalized response of five devices, I_DON and I_DOFF (I_{DS} at V_{GS} -0.8 and 0.4 V, respectively) were normalized against value at time 5 minutes, then average (and standard deviation) is depicted here. b) Artificial seawater stability test. Normalized response of four devices, I_DON and I_DOFF (I_{DS} at V_{GS} -0.8 and 0.4 V, respectively) were normalized against value at time 5 minutes, then average (and standard deviation) is depicted here.*

3.2 Nanoplastics test

To detect NPs in environmental samples, nowadays long and expensive processes are needed, some of the required steps are collection, filtration, optical characterization and identification (using spectroscopic techniques like Raman spectroscopy) (Oliveria and Almeida, 2019). Thus the final goal of the project is to use our developed EG-FET as a fast and reliable sensor for NPs detection. To do so, first the effect that different concentrations of PS- NPs have on the device was studied. After stabilization of the device (92 minutes in DI-water), six concentrations of PS were tested. Transfer curves were taken every 5 minutes and then I_DON and I_DOFF were normalized using equations 3 and 4. In figure 3a, transfer curves of one device are depicted; for clarity, only few time points are shown (5 and 92 minutes for DI-water, 12 minutes for each PS concentration). As expected, I_DON with DI-water after 92 minutes decreased compared to the initial value. Upon addition of PS solution, I_DON was at first stable,

***Table 1:** Increase (n=5 for DI-water, n=4 for artificial seawater) of I_DON after 12 min of each PS concentration. Increase compared to the final value of I_DON during water stability (92 min). ON/OFF of one representative device (I_DON and I_DOFF at time 12 min of each concentration used).*

PS concentration	DI-water test		Artificial seawater test	
	I_DON increase (%)	ON/OFF	I_DON increase (%)	ON/OFF
0.05 mg/ml	9.31 (±7.3)	3.98	3.53 (±1.9)	9.33
0.1 mg/ml	8.93 (±10.2)	4.32	9.24 (±5.7)	9.53
0.25 mg/ml	25.92 (±12.5)	5.18	13.72 (±5.1)	9.67

0.5 mg/ml	45.52 (±13.8)	6.72	17.8 (±7.1)	9.57
1 mg/ml	66.28 (±16.1)	7.98	20.36 (±9.6)	9.28
2 mg/ml	80.40 (±20.4)	8.80	22.80 (±11.3)	9.04

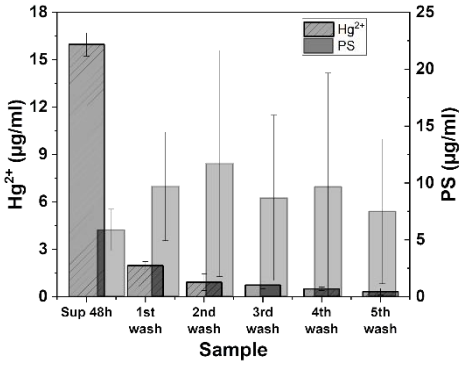

Figure 4: *PS-Hg^{2+} adsorption test. Concentrations of both Hg^{2+} and PS measured in the collected supernatants after each washing step.*

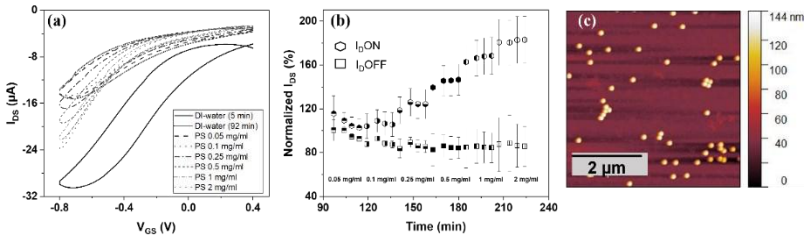

Figure 3: *DI-water and PS NPs test. a) Transfer curves of one EG-FET (only few time points are shown) V_{DS} -0.1 V, V_{GS} swept from 0.4 to -0.8 V (backward and forward direction). b) Normalized response of four devices. I_DON (V_{GS} -0.8*

and then started to increase with PS concentration 0.25 mg/ml, as it can be seen from the normalized response in figure 3b. In table 1, the increase of I_DON at each concentration is summarized. For the first two concentrations, the increase was low (less than 10%) and the standard deviation was in the same range, because of a high variability between devices. However, from PS 0.25 mg/ml, the increase was more remarked (25.92% ±12.5). With higher concentrations, I_DON showed an increase of 45.52(±13.8)% (0.5 mg/ml), 66.28(±16.1)% (1 mg/ml) and 80.4(±20.4)% (2 mg/ml). The increase of I_DON with increasing PS concentration, could indicate that PS NPs interacted with CNTs. Also, the hysteresis in transfer curves decreased during the test (clearly visible in figure 3a); PS NPs might act as a "passivation layer" (due to its hydrophobicity) and avoid water molecules to penetrate in CNTs network, which is one of the causes of high hysteresis in FETs (Kim et al, 2003). ON/OFF ratios were also compared and in table 1, values of one device are presented; higher PS NPs concentration was influencing the ON/OFF, which increased. For EG-FET with CNTs, ON/OFF values are in the range of $10\text{-}10^2$ A/A (Shkodra et al, 2021)), so our devices have comparable values. This parameter is often used in EG-FET based sensors, since it is directly influenced by the presence of the analyte of study. AFM images confirmed that PS NPs were adsorbed on the CNTs network, as shown in figure 3c. In addition, with XPS analysis, a difference in CNTs spectra before and after the test was noticed in the C1s peak. This could indicate a non-covalent interaction between CNTs and PS, possibly a π-π interaction. When artificial seawater was used as electrolyte, a general smaller increase in I_DON was noticed for each PS concentration, this is summarized in table 1. In this case, ON/OFF did not change with time, because also I_DOFF increased with time (similar to I_DON). This could be due to the presence of salts, which interfere with the current and the electric double layers, thus mitigating the effect of PS NPs.

3.3 PS-PET comparison

Same concentrations in particles/ml as for PS were tested, however only the three lower concentrations were tested because of low availability of the material. I_DON decreased with increasing concentrations of PET. For the first concentration ($9.09*10^{10}$ particles/ml) the decrease was -25.07%(±1.7) after 12 minutes; for concentration 2 ($1.82*10^{11}$ particles/ml) it was -36.19%(±4.0), for the third concentration ($4.55*10^{11}$ particles/ml) it was -57.66%(±5.6). This result shows an opposite trend compared to PS NPs (table 1). When artificial seawater (diluted 1:5 in DI-water) was used, the trend was similar, with a smaller total decrease (just like a total smaller increase was noticed with PS). The decrease was -0.97% (±1.39), -5.97%(±5.5) and -24.59%(±3.9) for the three concentrations respectively. AFM images of one device after the test were taken, CNTs network is still clearly visible, however no NPs seem present. This indicates that, probably, there was no interaction between PET NPs and CNTs. This result confirms that CNTs interact with PS NPs, not because of their shape but also because of their chemical structure. This is important for the developed sensor, since one of the most difficult tasks in NPs detection is the chemical identification.

3.4 PS-contaminants complexes

In the environment, NPs interact with other molecules, such as organic and inorganic contaminants, for this reason we wanted to study how PS NPs interacted with known environmental contaminants. First, Hg^{2+} ions solution was tested. Concentration of both Hg^{2+} and PS in each collected supernatant were measured, to assess the amount of Hg^{2+} and PS lost in each washing step, and thus calculate the amount of Hg^{2+} adsorbed on PS NPs; in figure 4 they are shown. It can be seen that after the first centrifugation step (sample sup 48h), ions concentration in the supernatant was high, however in the following washing steps, very little amount of Hg^{2+} was present, meaning that the remaining Hg^{2+} ions were indeed strongly adsorbed by PS. Using equation 5, the binding capacity was measured; three replicates were done and the average binding capacity was 2.04%. To confirm this interaction, XPS analysis was performed on the final solution containing PS-Hg complexes. As

expected, Hg4f peak was present in the general XPS survey, along with C1s and O1s peak (which were present also in pure PS solution). Furthermore, in the O1s peak there was an important difference: in pure PS solution, C-O and C=O are the main peaks, while for PS-Hg (both before and after the washing steps) also Hg-O peak is present. This confirms the chemical adsorption of Hg on PS NPs surface. PS-Hg complexes will be tested on EG-FET devices, to compare them with pure PS NPs (some preliminary tests have already been performed, but now shown here). The same test performed with glyphosate, did not produce the same results. In fact, most of the glyphosate was recovered in the supernatant after the first centrifugation step (after 48h of adsorption), meaning that it was not adsorbed on PS surface. To overcome this problem, different test conditions will be tested in the future; changes will include time of incubation, temperature or pH of the solution.

4 Conclusions and Future Perspectives

We developed an EG-FET device that could be used to study the interaction between PS NPs and CNTs; this interaction led to an increase in the measured I_{DS}. We saw an increase in I_{DS} dependent on the concentration of PS NPs; starting from PS 0.25 mg/ml, a net increase could in fact be seen. A physical interaction was first confirmed by AFM images, where NPs were indeed present on CNTs network. Then a possible non-covalent interaction (thus chemical interaction) between PS and CNTs was hypothesized, after XPS spectra of CNTs before and after test with PS NPs were compared. Furthermore, CNTs did not interact in the same way with another type of NPs, PET; in this case no physical or chemical interaction was observed. Compared to the current detection methods (for NPs), our device is fast, easy to fabricate and less expensive. In addition, the main novelty of our device consists in using EG-FETs as sensor for this specific contaminant; nowadays many EG-FETs are being developed, however not many in the field of environmental monitoring and no EG-FET for NPs detection has been yet developed. However, the developed device cannot be considered specific for the desired analyte yet, which is an important aspect for sensors to be used for environmental monitoring.
Our efforts are now concentrated on using a specific biorecognition element, to be functionalized on the device and that would allow us to obtain specificity and selectivity towards PS. With such a device, studies comparing pure PS NPs solution and PS-contaminants complexes solution will be performed, to assess how the devices interact with different solutions of NPs. Comparing the different solutions could give an indication on how the devices act on environmental samples, which is the final goal. Such a device would improve the current detection methods and especially improve them towards more standardized, fast and easy-to-use methods.

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Dietary Habits and Biogenic Amine Exposure in Mediterranean Populations: Insights from the Italian Aperitivo/Happy Hour Context

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This project is primarily focused on understanding the exposure of various Mediterranean populations to biogenic amines (BAs) through their dietary habits. To achieve this goal, an extensive survey was conducted to examine people's meal choices. Potentially hazardous meal occasions were identified, and specific food categories rich in biogenic amines were analyzed. The presented portion represents only a part of a larger research project, specifically focusing on the Italian population during aperitivo/happy hour occasions.

Abitudini alimentari ed esposizione alle amine biogene in popolazioni meditarranee: Approfondimenti sulla popolazione italiana durante l'aperitivo

Questo progetto è incentrato sul comprendere l'esposizione di diverse popolazioni del bacino Mediterraneo alle amine biogene, per mezzo delle abitudini alimentari. Per raggiungere tale scopo è stata condotta una minuziosa indagine sulle scelte alimentari delle persone. I pasti possibilmente pericolosi (per il contenuto in amine biogene) sono stati individuati e le classi di alimenti contenenti tali composti, analizzati. Qui viene presentata una parte di un progetto più vasto, quella riferita alla popolazione italiana rispetto all'occasione dell'aperitivo/happy hour.

Keywords: dietary habits, histamine, tyramine, exposure

1. Introduction

1. Before setting the risk exposure to BAs for distinct populations, several considerations were made.
2. Revise the available literature about the BAs role and their negative and hazardous potential.
3. Understanding the diversity of BAs in foods and beverages.
4. Focus on the population.
   A novel approach for gathering real (the closest to the reality) data on dietary habits, especially about food and beverages combinations, servings eaten, and frequency was considered. Moreover, this approach has led to know about hindering factors that may augment the effect of BAs, such as drugs assumption and others.
5. Analyze all this information together regarding specific meal occasions of the day.

2. The state of the art about BAs

The amino compounds included in the group of biogenic amines constitute a broad class of molecules involved in numerous activities within human metabolism. Primarily, they act as signaling molecules and regulate metabolic and hormone-related responses. They are generally recognized as safe and endogenously produced for the purposes briefly mentioned (Martuscelli et al., 2020). However, biogenic amines can become hazardous when the detoxification systems responsible for their management, namely monoamine oxidase and diamine oxidase (MAO, DAO), are overloaded or compromised,

BAs related symptoms	Manifestations	Recognized conditions
Respiratory	Asthma, rhinitis, general difficulties	Sgombroid syndorme,
Gastro-intestinal	Abdominal pain, nausea, diarrhea	Fish poisoning,
Dermatological	Cutaneous rash, urticaria, flush	Cheese reaction,
Headhache	Migraines (different severity)	Wine allergy,
Heart related symptoms	Palpitations, blood pressure disorders	Histamine allergy, Histamine intolerance

Figure 1 *BAs related symptoms. A summary of the main symptoms and known conditions.*

resulting in various symptoms (Figure 2). Due to their direct association with cellular metabolism, biogenic amines are commonly found in animal and plant tissues, as well as in microorganisms. The latter can convert amino acids into biogenic amines through specific enzymes called decarboxylases (Schirone et al., 2022). Many of these species are of technological interest in food production. Salami, cheeses, a wide range of other fermented products, and certain alcoholic beverages are produced through fermentation (Ashaolu et al., 2021). These items are popular worldwide and are prominent in the composition of Mediterranean countries' aperitivo/happy hour. The presence of biogenic amines in foods and beverages may pose a health hazard for consumers, leading to various symptoms of varying severity depending on specific patterns (Figure 2). These patterns can be categorized into personal health factors, food quality, and choice/combination factors. Naturally, these factors can coexist and collaborate to amplify the final effect (Sànchez Pérez et al., 2022).
Not all biogenic amines can be classified as toxic. Furthermore, due to the aforementioned reasons, establishing a defined hazard for everyday diets presents challenges. Multiple factors interact, and individual responsibility regarding the amount consumed daily can make any meal potentially capable of causing symptoms. Over the years, significant efforts have been made by the scientific community and institutions such as the European Food Safety Authority (EFSA). Currently, we have thresholds for histamine and tyramine and recognized pathologies primarily associated with these two amines (EFSA 2011; Commission Regulation (EC) 2073/2005). They are indeed toxic, and histamine, in particular, is known for its connection to certain fish species and their derived products.

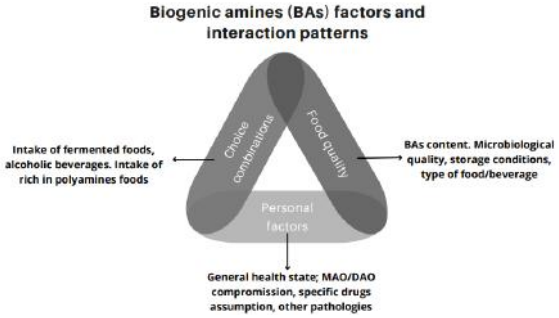


Figure 2 *BAs contributing factors, patterns, and interaction.*

3. Materials and methods

An important step of this project regarded the redaction of a survey to collect people answers, understanding how they compose their aperitivo/happy hour, and get frequency of consumption. This part was achieved with Google forms by Google platform. Surveys were primarily shared with students and personnel from the departments of biosciences and food technology, and communication sciences of the University of Teramo. Then, spread on social media to recruit a higher number of participants. Survey correctness was assessed by an internal validation with experts, then submitted to the advice of the Ethical Committee for the provinces of Teramo and L'Aquila before spreading. After receiving the positive statement, the survey was launched on-line. The elapse of time was of one month September-October 2022. A total of 424 valid responses was collected.
Contents of BAs from foods and beverages were in part determined ex novo, from several food matrices, and taken from internal database especially for fermented (animal-origin) foods. Experimental data were determined according to Latorre-Moratalla et al., (2009). Different classes of foods and beverages were analyzed and divided into meat-based foods, cheese and milk derived products, vegetables-based foods, seafoods and fish products, and alcoholic beverages. Mostly of the items indagated are known for their medium/high content of BAs and especially of histamine and tyramine; others were put because no data are available or few references report them. From a methodological point of view, we restricted the exploration about exposure to histamine and tyramine since they have established threshold limits to which compare. The landmark was the work of EFSA (2011) reporting for tyramine the thresholds of 6 mg/meal/person for patients treated with inhibitors of the enzyme monoaminoxidase (MAOI) drugs, 50 mg/meal/person for patients receiving third generation of MAOI drugs, so called RIMA (reversible inhibitors of MAO-A); and 600 mg/meal/person for healthy individuals. For histamine, the safe threshold considered for healthy population was 25 mg/meal/person as the most conservative level.

Calculation of exposure was done for those classes of products more chosen from respondents. Various probability distributions were fitted using @Risk 7.0 (Palisade Corporation, NewField, NY). The goodness of fit was evaluated using the Chi-square (χ^2) test. The best-fitting distributions describing tyramine or histamine contents and the consumption were selected as an input for the assessment of the exposure to these compounds by the probabilistic estimation using the Monte Carlo simulation technique with 10,000 iterations. Exposure consisted of crossing quantities of product eaten per occasion (g/aperitivo) with contents of histamine and tyramine (mg/kg of product) and summing all the products eaten for that occasion.

4. Results and discussion

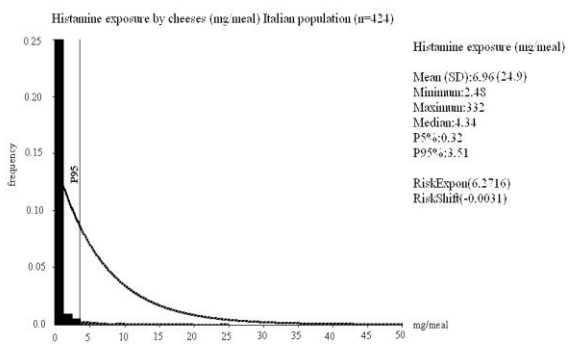
Based on the survey results, we can outline the profile of the average respondent. Women make up the majority of the sample, accounting for 66.4% with an average age of 30.09 ± 11.7 years. The majority of respondents (88.9%) follow an omnivorous dietary pattern. Among them, 21.4% report having allergies or intolerances, with lactose intolerance being the most prevalent (9.7%). Regarding medication, 17.6% of the participants regularly take birth control drugs (16 out of 71 subjects), antihypertensives (11 out of 71 subjects), and antihistamines (10 out of 71 subjects).

When it comes to aperitivos, meat-based foods and dairy products are the preferred choices. Specifically, the most popular meat-based foods fall into the category of "ripened not fermented or cooked not fermented foods" and "fermented uncooked products." For dairy products, "ripened, fermented cheeses" are the top choice. The first result regards the average intake of some products; 28.22 g ± 15.28 for 0.89 times a week for fermented uncooked products and 26.08 g ±18.32 for 1.81 times a week for fermented cheeses. Nuts are the most consumed vegetable-based option. In terms of beverages, wines (red, white, and rosé) and beer are equally favored. For certain food categories that do not contain histamine and/or tyramine (or contain them in reduced quantities), exposures were not calculated. Salami (fermented uncooked items), fermented cheeses, wines, and beers are instead discussed here.

The calculated exposure indicates a low probability of being exposed to histamine and tyramine through the consumption of non-cooked fermented products and fermented cheeses. Graphs illustrate the distribution of amine contents (mg/kg) versus frequency of occurrence. Examples of graphs for histamine and tyramine in fermented cheeses and beer are shown (Figure 3-4). The frequency of being exposed to tyramine through the consumption of fermented meat-based products is limited, with many iterations simulating a frequency value close to or equal to 0 mg/kg in 95% (P95) of the cases calculated. As for histamine exposure, the sample falls within a range of 0-3.51 mg/kg (mean value: 7.67±2.09). Again, many iterations yield a probability close to or equal to 0. For cheeses, the exposure to tyramine ranges from 0-277 mg/kg (mean value: 42.3±7.7). Regarding histamine, which generally has lower contents, the analyzed population (P95%) is exposed to a range of 0-3.51 mg/kg (mean value: 6.96±).

In general, alcoholic beverages are popular, with beer being more consumed than wine, averaging 433 mL±275. Red wine is preferred over white and rosé. The consumption rates are approximately 105 mL±73 for red wine and 133 mL±89.6 for white/rosé wine. Specifically, beer consumption leads to an exposure to tyramine ranging from 0-150.2 mg/meal (mean value: 27.54±53.4) and histamine ranging from 0-25.2 mg/meal (mean value: 4.48±9.6). For red wine, the range for tyramine is 0-10.94 mg/meal (mean value: 2.76±3.06) and for histamine, it is 0-8.94 mg/meal (mean value: 1.13±4.6). White and rosé wines have the following distributions: tyramine 0-12.9 mg/meal (mean value: 0.91)

Thus, if considering a common aperitivo (according to responses collected) it can be affirmed that an Italian consumer is possibly ingesting about 102.26 mg\meal of tyramine and 19.11 mg\meal of histamine for an aperitivo composed of 28.22 g of fermented uncooked products, 26.08 g of fermented cheeses and 433 mL of beer.



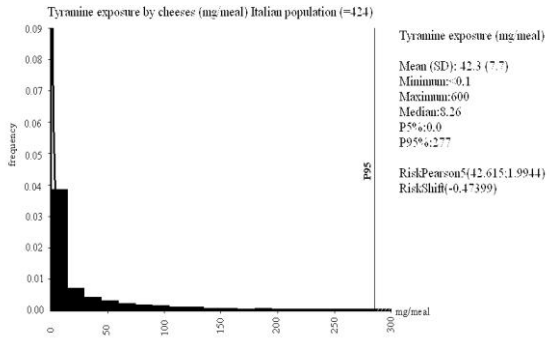


Figure 3 *Histamine and tyramine exposure by fermented cheese consumption during aperitivo with statistics of the distribution.*

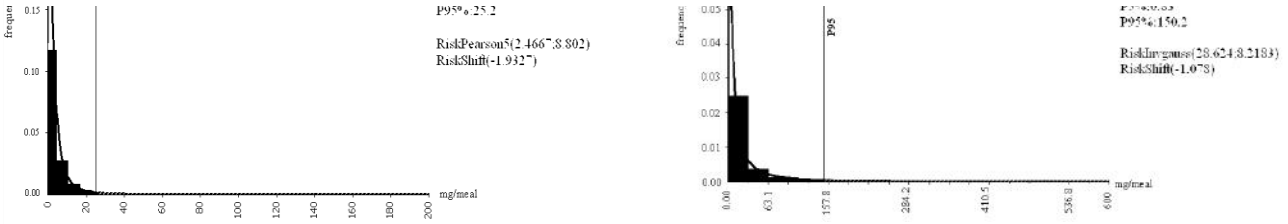


Figure 4 *Histamine and tyramine exposure by beer consumption during aperitivo with statistics of the distributions.*

5. Conclusions and future perspectives

As discussed, the concern about biogenic amines remains present in our lives like a continuous low-volume alarm. On one hand, limited legislation is in force to monitor histamine in some fishery products; on the other hand, exposure to hazardous ingestions in daily life is very limited, as previously shown. The problem with biogenic amines is that a great variability dominates the entire framework. Food samples may contain extremely different contents of several amines, as confirmed by the literature and this study. Moreover, the severity of symptoms covers such a wide diversity and may often be wrongly attributed to other conditions or generally dismissed as an allergy. In addition, these symptoms are usually very fast and transient, acting with tremendous individual specificity.

Recently, the difficulty of modulating the diet for allergic subjects has also been highlighted. The total elimination of fermented/suspicious foods may not be enough, as diverse physiological factors interact, and the human microbiome can enhance the production of some BAs. These compounds are stable under various conditions (temperature, pH, salt concentration), and those safe products (free of histamine and tyramine) can provide polyamines, which are widely distributed and can amplify the action of histamine and tyramine traces.

The most significant issue related to exposure to biogenic amines is still linked to people's habits and the difficulty in reporting accurate consumption data. The survey used in this study was carefully designed to capture the most realistic situation, accompanying each question with common foods and tableware to convert measurements perfectly into grams and milliliters. However, it is impossible to precisely match what an individual actually consumes during a meal.

Even though the data reported here depict a safe scenario, it must be considered that only one meal (the aperitivo) contributes a significant load of biogenic amines, especially when considering that only histamine and tyramine are calculated. As mentioned, other biogenic amines are not directly toxic, but their detoxification may simply require extra work from the enzymes involved, unless they are compromised due to specific conditions, or they may collaborate with histamine and tyramine, thereby increasing their hazardousness.

The general conclusion is that consumers should be advised of this potential hazard. This responsibility may primarily lie with medical doctors, but food producers could consider innovative ways to communicate about this reality, especially for fermented products or those already regulated by European law. This should not be seen as a negative aspect but, on the contrary, could become a qualitative enhancement of a product. The presented research also provides an overview of the general quality status of the analyzed samples, and as can be seen, all samples were obtained under optimal hygienic conditions.

Future steps involve observing other Mediterranean countries, primarily Spain, and investigating the snacking habits of children and toddlers throughout the day, shifting attention to other foods and beverages.

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Alternative Strategies for the Development of High-Nutritional-Value Products from Cereals and Pulses

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This PhD thesis aimed at improving the nutritional value of cereals and pulses-based food through different strategies as: using the natural modifications occurring during germination; exploiting underutilized crops; recover and upcycling food industry by-products with the possible implementation of high-power low-frequency ultrasound technology (US).

Strategie alternative per lo sviluppo di prodotti alimentari ad alto valore nutrizionale da cereali e legumi

Questa tesi di dottorato ha riguardato il miglioramento del valore nutrizionale degli alimenti a base di cereali e legumi attraverso strategie diverse, quali: le naturali modificazioni che si verificano durante la germinazione; lo sfruttamento delle colture sottoutilizzate; il recupero e il riciclo dei sottoprodotti dell'industria alimentare con l'eventuale adozione degli ultrasuoni a bassa frequenza.

Key words: pulses; legumes; cereals; ultrasound; germination; sprouting; by-product; sidestream.

1. Introduction

Consumers interest towards alternative products with high nutritional value is pushing the food industry to fulfil this demand by identifying and introducing new food resources. Lupin, a pulse crop with thrifty agronomic requirements, represents an excellent source for human nutrition being rich in proteins, lipids (mainly unsaturated fatty acids) and antioxidant compounds (Briceño Berru *et al*., 2021; Estivi *et al*., 2022a). Lupin utilisation is hampered by the limited number of studies on its composition and technological characteristics, as well as the lack of improved varieties suitable for cultivation in the main lupin cropping areas. Furthermore, despite their excellent nutritional composition, lupin seeds must be debittered before consumption to remove toxic alkaloids, a further limitation to their diffusion because debittering is a water-intensive and protracted process, which lasts up to six days (Estivi *et al*., 2022b).
Peas occupy a prominent place among vegetables due to their high content in protein (23–25%), digestible starch (50%), soluble sugars (5%), fibre, vitamins A and C, calcium and phosphorus (Sharma *et al*., 2013). However, industrial processing discards from 5 to 25% of the harvest as by-product (i.e., pods, husks and broken, dark or stained seeds; Conserve Italia Scarl., Italy), still rich in protein, dietary fibre, polyphenols and other molecules (e.g., peptides and lectins) with antioxidant or antimicrobial activities (Mateos-Aparacio *et al*., 2010). The high amount of protein has pushed the food industry to try to upcycle legume by-products by incorporating them into various kinds of food (e.g., high protein pasta, chips, hamburger patties, nuggets, beverages, baby food, imitation cheese, whipped toppings, soy milk and baked goods; Boye *et al.*, 2010). A popular way to exploit them is also the extraction of valuable fractions. Several techniques are employed to obtain protein concentrates and isolates from pea flour, including alkaline wet extraction/isoelectric precipitation (IEP) without or with ultrafiltration (Qiaoyun *et al*., 2017; Vogelsang-O'Dwyer *et al*., 2021), dry fractionation with possible tribo-electric separation (Wang *et al.*, 2015), salt extraction, micellization and gentle fractionation, or hybrid wet/dry approaches (Geerts *et al.*, 2017). The wet techniques can be coupled with high-power low-frequency ultrasound technology, whose capability to improve extraction yield and duration is well-documented (Estivi *et al.*, 2022c).
In recent years, alongside vegan and vegetarian products (Kumar *et al*., 2017), the demand for functional foods enriched with bioactive compounds of plant origin has gradually increased, driven by consumers awareness of the close relationship between nutrition and health. However, the deficiency of vitamin B12 intake emerged as a major risk in strictly vegetable-based diets, naturally devoid of it (Chamlagain *et al*., 2015). Moreover, the vast variety of commercial B12 supplements from algae, often containing the inactive pseudovitamin, contributes to mislead the consumers (van den Oever and Mayer, 2022). Fermentation with B12-producing *Propionibacterium freudenreichii* strains has proved to be a feasible strategy for *in-situ* enrichment of cereal and legume ingredients (Xie *et al*., 2021).
In accordance with the PhD thesis project previously described (Estivi, 2021), this oral communication reports the main results of the following activities directed to:
A1) study the effect of controlled germination on lipophilic antioxidants (i.e., tocols and carotenoids) and

colour in Andean lupin seeds;

A2) characterize the water-debittered seeds of 33 Andean ecotypes of *Lupinus mutabilis*, originating from different regions of Peru, for free phenolic compounds and perform a preliminary investigation of FT-NIR suitability as a fast, reliable and non-destructive approach to assess their antioxidant properties;

A3) develop and fine-tune a method for fast and water-efficient debittering of white lupin seeds;

A4) evaluate the impact of the debittering method on the antioxidants of white lupin seeds;

B1) develop and optimise an ultrasound-assisted alkaline extraction of protein to upcycle the by-product from canned green peas production (pea waste, hereafter);

B2) evaluate the pea waste as a culture medium for synthesizing vitamin B12 by *P. freudenreichii* fermentation and prepare a B12-rich bread with fermented material.

2. Materials and Methods

A1) Two Andean lupin cultivars (*Lupinus mutabilis* Sweet) were analysed before and after germination in the dark for two, four and six days. Colour coordinates (*L**, *a**, *b**) were evaluated by a tristimulus colorimeter, while tocols and carotenoids were extracted by saponification (Panfili *et al*., 2003) and quantified by HPLC (Brandolini *et al*., 2022).

A2) 33 Andean ecotypes of *L. mutabilis* and five varieties belonging to *L. luteus*, *L. angustifolius* and *L. albus*, as controls, were analysed to assess their free phenolic compounds content by RP-HPLC (Brandolini *et al*., 2022). The acquisition of FT-NIR spectra was performed as well.

A3) Two lots of white lupin (*Lupinus albus*) seeds were used in different trials to optimize the experimental debittering method and to assess the influence of sonication (with and without the employment of US), solvent (water, solutions of salt or citric acid) and treatment time. The effectiveness of debittering was evaluated by extraction and titration of the residual alkaloids (von Baer *et al*., 1979) and evaluation of the bitterness by e-tongue (Marengo *et al*., 2016). Two debittering reference methods (Córdova Ramos *et al*., 2020; Villacrés *et al*., 2020) and four commercial lupin snacks were used as controls.

A4) The very same materials produced and analysed in the A3 activity were characterized for their content in tocopherols, carotenoids and phenolic compounds (soluble free, soluble conjugated and bound fractions) by HPLC as previously detailed.

B1) The by-products of the canned peas production line were sampled on three different days and stored at -20 °C. According to the Design of Experiment (DoE) technique, trials were carried out to fine tune ultrasound-assisted alkaline extraction of protein followed by isoelectric precipitation from thawed by-product. Protein concentrate, dried by-product and a commercial pea flour were characterized for protein content (Kjeldahl), water activity, colour, water and oil retention capacity, gelling capacity, foaming capacity and stability, emulsifying activity, protein pattern by SDS-PAGE, total bacterial count, total lactic acid bacteria, *Enterobacteriaceae*, moulds and yeasts.

B2) The dried pea waste described in the B1 activity was fermented with the following procedure, established during several preliminary trials: 500 g of a 15% batter were prepared mixing dried pea waste with MilliQ water; the pH was adjusted to 6 with 10 M NaOH and the batter was pasteurized at 70 °C for 20 min and then aseptically inoculated with *P. freudenreichii subsp. freudenreichii* 282 culture to reach a cells concentration of approximately 9 log CFU/g. The material was incubated at 30 °C for 72 h with constant shaking. Fermentation was performed in two independent repetitions. Six lots of bread were baked according to Edelmann *et al*. (2016), including two controls (with and without addition of non-fermented pea waste batter) and four enriched lots (with 15 and 20% for each of two batches of fermented batter). Vitamin B12 and organic acids were quantified as outlined by Chamlagain *et al*. (2015) and Xie *et al*. (2018), respectively. Propionibacteria, lactic acid bacteria and *Enterobacteriaceae* were enumerated by plate count in agarised YEL, MRS and VRBG, respectively. The baking loss was determined gravimetrically; bread volume and crumb texture were determined by Volscan and texture analyser (Stable Microsystems), respectively.

3. Results and Discussion

A1) Germination significantly affected all the examined characteristics (Estivi *et al*., 2022d). Luminosity (*L**) showed an uncertain pattern as the germination time increased, but minor reduction in *a** and relevant growth in *b** indicated yellow-greenish compounds formation. No tocotrienols were found and the most abundant tocol was γ-tocopherol. Although total tocols were almost unchanged during germination, α-tocopherol increased from 0.7 to 74.8 mg/kg dry matter (DM) after six days, while γ-tocopherol (Fig. 1) decreased. The γ homologue is the precursor of α-tocopherol, hence we suggested that germination triggered their conversion, improving 4.3-fold lupin flour biological activity. The most abundant carotenoid was lutein, but (α+β)-carotene, β-cryptoxanthin and zeaxanthin were identified as well. Total carotenoids increased 12-fold with germination time, from 1.8 to 22.3 mg/kg DM (Fig. 1).The (α+β)-

carotene, precursor of vitamin A, showed the greatest growth rate; β-cryptoxanthin and zeaxanthin, originally below or close to the detection limit, exhibited detectable amounts after six days (1 and 1.5 mg/kg DM, respectively); lutein continued to prevail, with a final amount of 13.6 mg/kg DM. Germinating seeds in the dark proved to be a viable and effective technique to significantly improve the nutritional properties of Andean lupin.

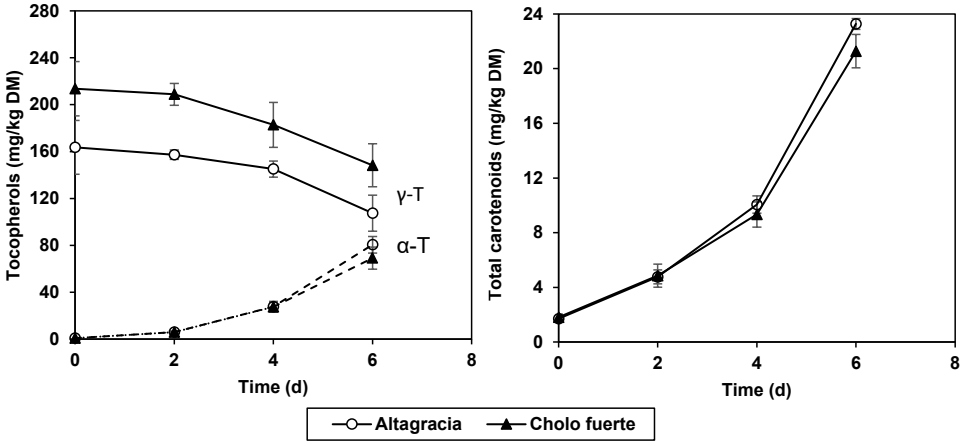

Figure 1 *Changes in tocopherols and carotenoids content in two Andean lupin accession, Altagracia (circles) and Cholo fuerte (triangles), during controlled germination up to 6th day. Error bars indicate the standard deviations.*

A2) The total free phenolics of *L. mutabilis* were mostly (85.5–99.6%) flavonoids (genistein and genistein derivatives, apigenin, catechin and naringenin). Other compounds, detected in low quantities, were phenylethanoids (tyrosol and tyrosol derivative) and phenolic acids (cinnamic acid derivatives). The total free phenolic concentration ranged from 340.8 (cv. Churibamba) to 1393.3 mg/kg DM (cv. H6 INIA BP) exceeding that of controls (6.8–31.3 mg/kg DM). A relationship between free phenolic compounds and spectral bands was established by FT-NIR, paving the way for a fast, reliable and non-destructive approach to lupin seeds characterization. Even after debittering, lupin flours maintained high free phenolic concentrations and antioxidant capacity.

A3) The sonication did not accelerate debittering, while the sodium chloride and citric acid solutions significantly shortened debittering time, reduced water consumption and decreased alkaloid content to commercial values (0.31–1.03 g/kg DM). Debittering with a 1% citric acid solution saved 88 h and 65 L water/kg dry lupin compared to the water control method, and 13 h and 31 L water/kg dry lupin compared to the salt solution control method. The electronic tongue grouped the experimental and commercial samples in well-defined clusters; bitter and umami tastes were the main factors, well correlated with alkaloid content. The proposed procedure, either with citric acid or sodium chloride, could be easily adopted by the industry to reduce time and costs of lupin debittering.

A4) The sonication decreased the content of carotenoids and soluble-free phenolics but did not influence tocopherols or soluble-conjugated and insoluble-bound phenolic compounds. Nevertheless, the debittered lupins showed interesting quantities of tocopherols (172.8–241.3 mg/kg DM), carotenoids (10.9–25.1 mg/kg DM), and soluble-free (106.9–361.1 mg/kg DM), soluble-conjugated (93.9–118.9 mg/kg DM), and insoluble-bound (59.2–156.7 mg/kg DM) phenolic compounds. Using citric acid or sodium chloride solution preserved in a better way the soluble-free phenolics likely due to the reduction in treatment times (Estivi *et al*., 2022e).

B1) The optimised extraction conditions were: ratio water/by-product, 20 mL/g; pH, 11; amplitude, 80 µm; time, 2 x 30 min; on/off cycle, 5/5 s; temperature, 25 °C. Table 1 compares the efficiency of ultrasound-assisted extraction *vs.* magnetic stirring and reports the characterisation of commercial pea flour, dried pea waste and protein concentrate. The extraction yield of the optimized process (66.6%) was comparable with that (62.6-76.7%) reported by Stone *et al*. (2015) from defatted pea flour with magnetic stirring only but was inferior to those (82.6% with ultrasonication and 60% with magnetic stirring) of Wang *et al*. (2020), possibly due to leaching of low molecular weight peptides formed during pre-sampling fermentation. This was confirmed by total microbial count (7.32 log CFU/g) and presence of smeared bands in the SDS-PAGE. Overall, the ultrasonication increased 3-fold the protein recovery yield, reducing to 1/4 the extraction time, at the cost of a lower purity of the concentrate. Prestes Fallavena *et al*. (2022) and Thirunavookarasu *et al*. (2022) collected numerous evidence related to protein glycation mediated by high-power ultrasound and to the formation of complexes between protein (sonication-denatured) and simple sugars, oligosaccharides or polysaccharides, including pectins. Thus, it can be hypothesized that the polysaccharides from the abundant soluble fibre of the pods formed soluble adducts

with protein, reducing the purity of the concentrate.

B2) To allow the growth of *Propionibacterium,* pea waste was pasteurized to reduce lactic acid bacteria and enterobacteria competition, and the excess of lactic acid was neutralized. In preliminary trials was observed that insufficient batter shaking led to accumulation of 40-42% vitamin as pseudo-B12; in fact, oxygen availability is crucial in the synthesis of 5,6-dimethylbenzimidazole, the lower ligand distinguishing the active form of B12 (Chamlagain *et al*., 2018). In Table 2 the main results are summarized. The fermented batters reached an average B12 amount of 208.9 ng/g fresh weight (FW) approximately equivalent to 1390 ng/g DM, a high value compared to the results reported by Xie *et al*. (2021) for eleven fermented batters from cereals and legume flours: 51–742 (mean 301) ng/g DM. Such a high B12 content made it possible to reach the remarkable average amounts of 35.8 and 51.7 ng/g FW in 15 and 20%-enriched breads. Given a recommend daily intake equal to 2–2.4 µg (Chamlagain *et al*., 2018), about 40 to 70 g/d enriched bread would provide enough vitamin. Enriching the dough caused moderate loss in bread volume and increase in crumb hardness but did not affect the overall quality in a substantial way.

Table 1 *Comparison of ultrasound-assisted extraction vs. magnetic stirring and characterisation of commercial pea flour, dried pea waste and protein concentrate.*

	Magnetic stirring	Ultrasonication	
Time	2 x 2 h	2 x 30 min	
Yield (%)	21.5±0.9	66.6±1.6	
Protein (g/100 g)	86.4±0.3	68.4±0.2	
	Pea flour	**Dried by-product**	**Protein concentrate**
Protein (g/100 g DM)	$25.09^{b} \pm 0.08$	$24.71^{b} \pm 0.71$	$74.86^{a} \pm 0.32$
a_w	$0.530^{a} \pm 0.004$	$0.320^{c} \pm 0.002$	$0.510^{b} \pm 0.002$
Colour coordinates			
L*	$86.50^{a} \pm 0.30$	$54.47^{b} \pm 0.83$	$40.43^{c} \pm 0.35$
a*	$-9.63^{b} \pm 0.21$	$-4.10^{a} \pm 0.46$	$-3.50^{a} \pm 0.30$
b*	$16.90^{b} \pm 0.35$	$19.17^{a} \pm 0.50$	$15.43^{c} \pm 0.55$
Water holding capacity (g H_2O/g)	$1.23^{c} \pm 0.34$	$4.19^{a} \pm 0.28$	$2.13^{b} \pm 0.22$
Oil holding capacity (g oil/g)	$1.57^{b} \pm 0.08$	$2.24^{a} \pm 0.16$	$1.68^{b} \pm 0.22$
Gelling capacity (g/100 mL)	$21.52^{b} \pm 0.28$	$16.09^{c} \pm 0.99$	$28.67^{a} \pm 1.62$
Foaming capacity (%)	$69.18^{a} \pm 1.39$	$21.59^{c} \pm 1.36$	$28.46^{b} \pm 1.39$
Foam stability (%)	$49.55^{a} \pm 0.01$	$15.21^{b} \pm 2.08$	$6.38^{c} \pm 1.39$
Emulsifying activity (%)	63.26 ± 1.15	62.20 ± 1.28	60.89 ± 1.07

Table 2 *Pea waste batters and enriched breads characterisation: B12 levels, microbial counts, organic acids, baking loss, specific volume and crumb hardness.*

Batter batches	**Fermentation time (h)**	**B12 (ng/g)**	**Propioni-bacteria**	**Lactic acid bacteria**	**Acetic acid (mg/g)**	**Propionic acid (mg/g)**
1	0		8.64±0.09	3.13±0.12		
1	72	211.3^{a}±22.9	9.93±0.04	4.86±0.20	2.81±0.01	4.05±0.01
2	0		8.63±0.04	3.76±0.07		
2	72	206.5^{a}±25.8	9.98±0.01	5.31±0.51	2.78±0.01	4.03±0.01
Bread batches	**Enrichment (%)**	**B12 (ng/g)**	**Baking loss (%)**	**Volume (ml)**	**Specific volume (ml/g)**	**Hardness (N)**
Control	0		13.3^{a} ±0.5	381.9^{a}±3.6	2.90^{a}±0.03	14.0^{bc}±1.1
Control-NF	20		12.2^{d}±0.7	379.9^{a}±1.5	2.88^{b}±0.03	11.9 ^{c}±0.8
1a	15	39.7^{b}±0.5	12.3^{cd}±0.3	379.6^{a}±1.9	2.89^{b}±0.02	16.2^{ab}±1.6
2a	15	32.0^{b}±4.1	13.0^{ab}±0.2	367.2^{b}±2.0	2.82^{c}±0.01	14.1^{bc}±1.1
1b	20	52.3^{a}±6.3	12.5^{bcd}±0.2	358.1^{c}±3.2	2.73^{d}±0.03	17.9 ^{a}±2.0
2b	20	51.1^{a}±3.5	13.0^{abc}±0.2	351.3^{d}±2.1	2.69^{d}±0.01	16.3^{ab}±0.6

4. Conclusions and Future Perspectives

Lupin was confirmed as a promising source of antioxidants, whose composition can be further improved by germinating seeds, selecting the most promising accessions for cultivation, and adopting optimised debittering to limit leaching of valuable compounds. The proposed procedure, either with citric acid or sodium chloride, could easily be adopted by the industry to reduce time, water consumption and costs of lupin debittering. Further studies should consider the incorporation of germinated lupin flour in food. Two approaches were attempted for the first time to upcycle the pea canning by-product: i) protein extraction based on ultrasound technology was developed; ii) waste fermentation for synthesising B12 proved to be feasible, although microbial alteration was identified as a limit, emphasising the importance of in-line processing. The fermented material was successfully added to the dough to obtain a bread rich in vitamin B12 (35.7–51.7 ng/g), without affecting leavening and volume development.

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Sensory methods ensuring authenticity and fostering Mediterranean fish

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The aim of this PhD project is to study and foster Mediterranean fish products, ensuring authenticity and improving subjects' trust, by using sensory analysis. It was achieved through a multidisciplinary approach concerning the study of consumer behavior toward seafood products, and through the development and optimization of descriptive sensory methods to assess the authenticity and quality of Mediterranean seafood products. The results are focused on the reduction of fishing fraud and improving consumers' trust in fish products by studying all the useful aspects, both information on the traceability and authenticity of fish and sensory and nutritional features.

Metodi sensoriali per garantire l'autenticità e valorizzare i prodotti ittici del Mediterraneo

Il presente progetto di dottorato ha l'obiettivo di studiare e valorizzare i prodotti ittici del Mediterraneo, mediante l'impiego di metodologie sensoriali. L'obiettivo è stato raggiunto attraverso un approccio multidisciplinare riguardante lo studio del comportamento del consumatore nei riguardi dei prodotti ittici, e attraverso lo sviluppo e l'ottimizzazione di metodi sensoriali descrittivi capaci di garantire autenticità e qualità ai prodotti ittici del Mediterraneo. I risultati si concentrano sulla riduzione delle frodi nel settore della pesca e sul miglioramento della fiducia dei consumatori nei confronti dei prodotti ittici, studiando tutti gli aspetti utili, sia riguardo le informazioni sulla tracciabilità e l'autenticità del pesce, sia le caratteristiche sensoriali e nutrizionali
Key words: Fish quality, reduction of fishing fraud, consumer science, descriptive sensory analysis.

1. Introduction

Fish products are widely consumed all around the world thanks to their nutritional composition rich in protein, long-chain fatty acids (EPA and DHA), and vitamins, and poor in carbohydrates (Carlucci et al., 2015). Nowadays, global aquatic food consumption is estimated at 158 million tonnes, up from 28 million tonnes in 1961. Since 1961, consumption has increased by approximately 3% annually, this increase is due both to an increase in per capita consumption, but also to an average increase in world population (FAO, 2022). Increased consumption of seafood has created jobs for the community (Shamsuzzaman et al., 2020), but is reducing natural fish stocks in the sea, and could be dangerous for the environment (Verbeke et al., 2007). Over the years, subjects have become more sensitive to issues of environmental sustainability and traceability of the products they buy (Pucci et al., 2020). Hence, some brands have joined sustainability and traceability programs to inform and guide subjects toward sustainable purchasing choices (Van Loo *et al.*, 2015). Moreover, the increasing trend in fish consumption increase fraud related to fish authenticity and quality, especially for less experienced subjects (Giusti et al., 2019). Finally, subjects are not always able to assess the quality of the species they buy, as not all the specific attributes of each fish species are known, and furthermore, there are no standard methods for assessing the freshness of fish. Thereby, this process can be difficult during purchase (Freitas et al., 2021). Therefore, this PhD project aimed to valorise Mediterranean fish products through a multidisciplinary approach concerning the study of consumer behavior toward seafood products, and through the development and optimization of descriptive sensory methods to assess the authenticity and quality of Mediterranean seafood products. To achieve the set objectives, the following activities were conducted:
A1) Study of subjects' behaviour towards fish products. This activity had a particular focus on fish product consumption habits, purchasing behaviour and the importance of fish traceability and sustainability.
A2) Consumer study to assess the effect of external variables provided to the subjects when purchasing or consuming fish products.
A3) Descriptive sensory analysis to attest to the authenticity of different fish species.
A4) Quality index method for assessing the quality and freshness of fish species during the purchase time.
A5) Study of subjects' behaviour in northern Europe. This activity was carried out during the PhD student's time abroad at Nofima Research center in Tromsø and Ås (Norway). The activity focused on the perceptions of Northern European subjects towards the traceability and sustainability of red king crab. In addition, a descriptive analysis test was carried out with a trained panel to test the differences between crabs from capture-based aquaculture and wild.

2. Materials and Methods

First, an online questionnaire (A1) was filled out by 2000 subjects from both Italy and Spain. The questionnaire collected information on fish consumption and purchasing habits (frequency of consumption, types of purchased products, places of purchasing, etc.) and the perception and importance of fish traceability and sustainability (1-7 Likert scale). Subjects were equally distributed for social-demographic information. Then, a consumer test (A2) was conducted with 100 Italian and 100 Spanish subjects, by using a blind-info procedure: in the first session the subjects evaluated a sample of bluefin tuna (*Thunnus thynnus,* both raw and cooked) and rated their liking for specific sensory characteristics, using a 9-point Likert scale (1: Extremely disliked; 9: Extremely liked), without sample information; in the second one, one week later, the same subjects performed the same evaluation but having been informed that the sample had been caught according to the terms of the Marine Stewardship Council (MSC) sustainability label. Subsequently, a classical descriptive analysis (QDA) was performed (A3) both of yellowfin tuna (*Thunnus albacares)* and bluefin tuna (*Thunnus thynnus*), identifying their peculiar sensory characteristics. Finally, Quality Index Method (QIM) (A4) was applied to assess the quality and freshness of fresh anchovies (*Engraulis encrasicolus*), stored by using a static electric field generator (SEF). Fresh anchovies were stored in polystyrene containers covered with melted ice and placed in two refrigerated cells at 4±1 °C. Only one of the two cells was equipped with the SEF generator. The anchovies from shelf-life day 1 to day 9 were evaluated by 9 assessors who used an optimized QIM protocol (Fiorile et al., 2023).
To assess the perception of traceability and sustainability by Northern European subjects, an online questionnaire was sent out. Subjects were shown pictures of red king crab in different settings and then a list of attributes was provided to them. The CATA method was used to collect responses to this questionnaire. Finally, again a QDA was carried out to characterise the two samples (capture-based aquaculture and wild) and to highlight the differences between them (A5).
The respondents to the online questionnaire were grouped for provenance (Italy and Spain), gender, and age (4 age groups) for 16 observations. Multifactorial analysis (MFA) was used to study the relationship between the observations and all the collected variables (A1). Paired sample t-test was used to find differences between the two experimental conditions in the consumer test (A2), to find differences between the two tuna species in the QDA (A3) and finally to find differences between the two storage conditions in the QIM (A4). Linear regression was used to describe the increment of QI during storage time for both trials (A4). The confidence level was 95% in every statistical test used. XLSTAT statistical software (v.2016.02, Addinsoft) was used for data analysis.
The data analysis of A5 is still in progress, thus the results are not illustrated here. In this manuscript, only the main results are discussed.

3. Results and discussion

3.1. Online questionnaire

Figure 1a is a representation of the subjects on the first two dimensions extracted by MFA accounting for 62.94% of the variance. Subjects were well separated in terms of provenance and age, indeed Spanish subjects are located in the upper part of the graph, while the Italian subjects are located in the lower part of the graph; as well as, the younger subjects are located in the left part of the graph, while the adults are in the right part of the graph. Figure 1b allows observing the variables which characterize the subjects and the associations among them. Adult Spanish subjects consume more fish than Italian ones, on the other hand, Italian subjects like fish more than Spanish. Also, adult Spanish subjects consume more fresh fish, while adult Italian subjects are more familiar with frozen and canned fish. Focusing the attention on sustainability and traceability, young subjects resulted more familiar with sustainability labels than adult ones, while the adults were more interested in traceability. Although subjects have become more sensitive to environmental issues (Pucci et al., 2020), our results confirm the low awareness of fish sustainability labels due to a lack of information on this topic, as reported by Garcìa-Herrero *et al*. (2019).

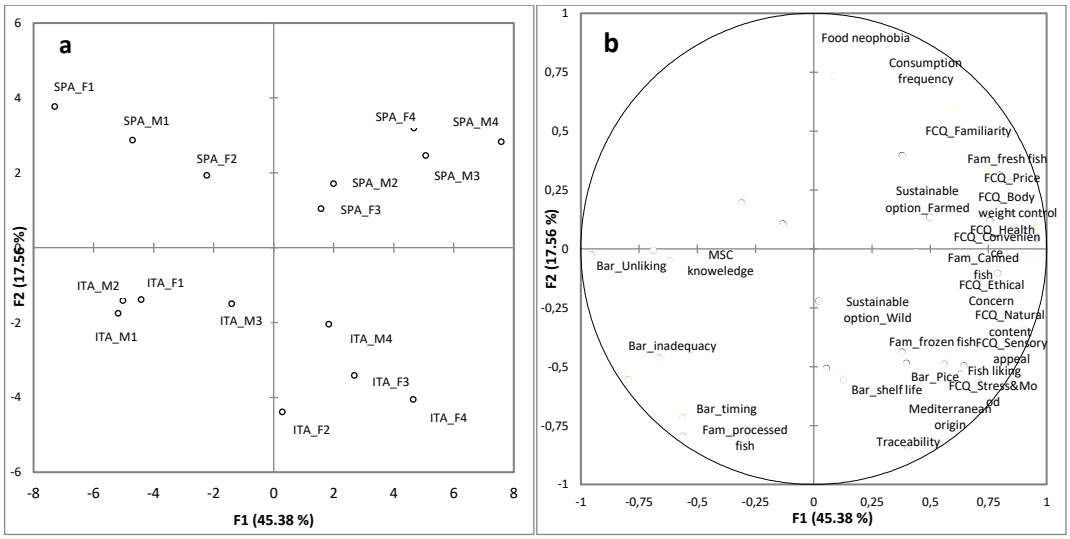

Figure 1- *Observation map (**a**) and Variables map (**b**) – MFA results*

3.2. Consumer test

The consumer test carried out on 100 Italian and 100 Spanish subjects showed that liking scores for the attributes assessed on both raw and cooked tuna samples increased when the sustainability label information was provided (Figure 2a-b). Specifically, statistical analysis showed significant differences in overall liking in Italy ($p \leq 0.05$), and in overall liking and appearance in Spain ($p \leq 0.05$).

Despite the subjects demonstrated to have a limited knowledge of sustainability labels and the different aspects they cover (Hoek et al., 2021), the sustainability label information increased the evaluated scores. As reported by other authors, the addition of external information (brand, labels) provided at the time of purchase can greatly influence product perceptions and choices (Olsen, 2003; Altintzoglou and Heide, 2016).

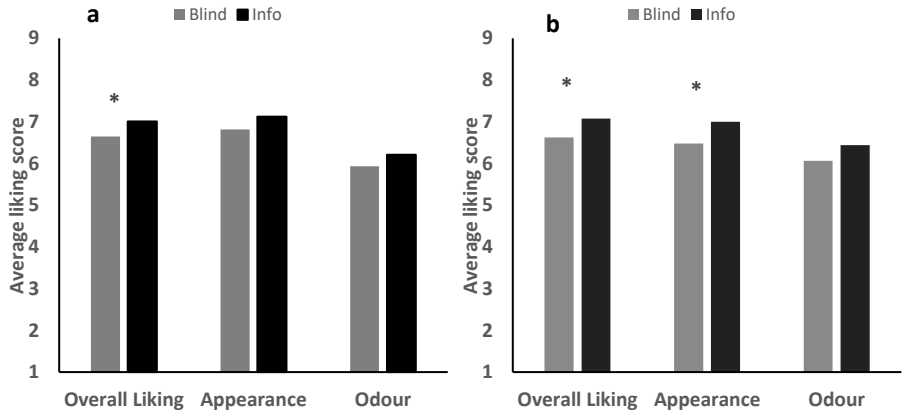

Figure 2- *Average liking score in Italy (**a**)and average liking score in Spain (**b**). Asterisks indicate significant differences (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)*

3.3. Descriptive analysis

Classical descriptive analysis (QDA) was useful to characterise and discriminate the two tuna species treated. Preliminary focus groups were needed to draw up a final list of five descriptive attributes for raw tuna and eleven for cooked tuna. The attributes were standardised for the two tuna species to have a single list.

Results showed that the two evaluated species were significantly different from each other in all the descriptive attributes when evaluated as raw ($p \leq 0.05$). The cooking process, on the other hand, weakened the differences between the two species, in fact, of the eleven attributes assessed, only five showed statistically significant differences ($p \leq 0.05$) (Figure 3a-b). The results of the descriptive analysis allowed the identify peculiar sensory for two tuna species and discriminate between them. Although they may seem similar, yellowfin tuna (*Thunnus albacares*) and bluefin tuna (*Thunnus thynnus*) have substantial nutritional and economic differences. As reported by Cutarelli et al. (2014) one of the possible fraud in the fisheries sector is misleading species substitution (e.g. less valuable species sold in place of more valuable species).

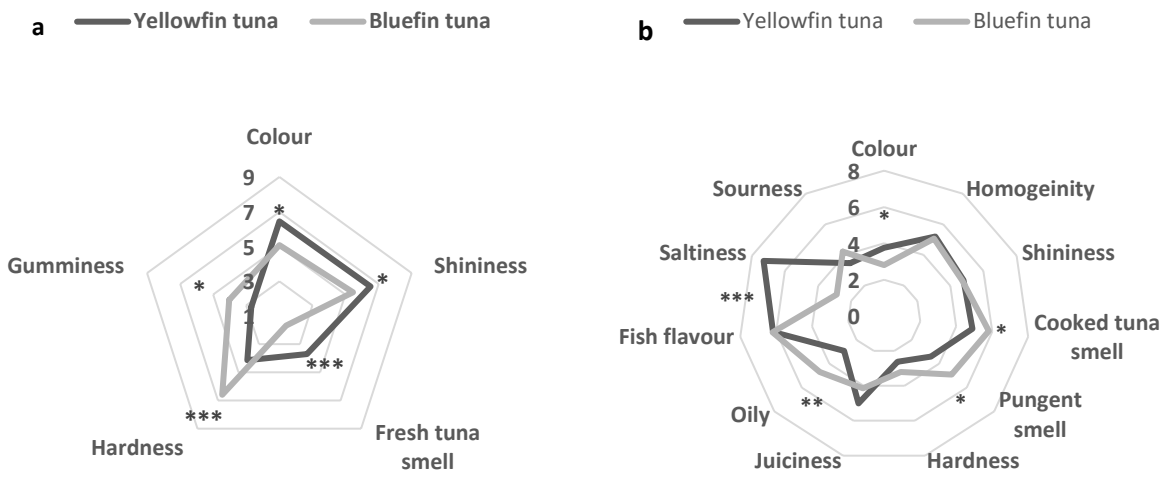

Figure 3-*Raw tuna evaluation (**a**)and Cooked tuna evaluation (**b**). Asterisks indicate significant differences (* p<0.05; ** p<0.01; *** p<0.001)*

3.4. Quality Index Method

This activity aimed to assess the freshness of anchovies using the QIM and to compare the evolution of the quality index (QI) over time between two different storage conditions: classic refrigeration and refrigeration with a static electric field (SEF). As reported in Figure 4, the maximum score achieved by the anchovies was 20 in the classic storage condition and 15 in the trial with SEF. Describing the QI increment by using linear regression (R^2=0.97 for the control sample, R^2=0.96 for SEF samples), as required by the QI method, results clearly showed that the SEF technology was able to reduce the QI score increment rate (slope = 2.0 day-1) compared to the control sample (slope = 2.5 day-2). The results obtained by anchovies preserved in the classical condition are consistent with other studies on anchovies' QIM (Massa, Manca and Yeannes, 2012; Pons-Sánchez-Cascado *et al.*, 2006). The improved condition of anchovies reported during storage with SEF is in agreement with other studies (Xanthakis *et al.*, 2013; Dalvi-Isfahan, Hamdami and Le-Bail, 2016). Moreover, as evidenced by the paired samples t-test, the two methods showed statistically significant differences ($p \leq 0.05$). There is a first difference after a three-day of shelf-life. But the differences are evident from day 7 onwards (Figure 4). Thus, since the scores of the two trials are similar for short shelf life, the preservative effect of the static electric field produced by SEF is more evident for longer shelf life.

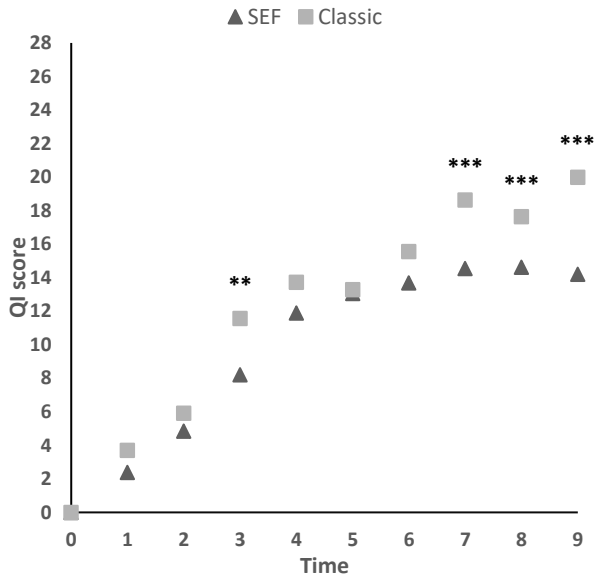

Figure 4-*QIM results, comparison between the two different trials. Asterisks indicate significant differences (* p<0.05; ** p<0.01; *** p<0.001)*

4. Conclusion and Future Perspectives

This PhD project aimed to foster Mediterranean fish products. To achieve this, activities started with an online questionnaire to find out current knowledge about fish products in two developed countries, where fishing is a source of income and part of the culinary culture. The results of the questionnaire reported information on the consumption habits of both populations concerning fish products, and the subjects' interest in fish traceability and sustainability emerged, even though the topic is not fully known to all subjects. Indeed, the consumer test showed how the addition of external information can influence the subjects' final judgment of liking. The descriptive analysis, on the other hand, can improve the knowledge of the fish species commonly found on the market. Finally, the quality index method showed itself to be easy to use in assessing the freshness of fresh anchovies. Furthermore, by combining normal refrigeration with a static electric field, the quality index can be reduced and thus the shelf life of anchovies can be increased. Future activities can be concentrated on other Mediterranean fish species, and dissemination campaigns should be promoted to better inform people about what they are buying, and to promote the purchase of traceable, sustainable fish and above all avoid fraud.

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Wine stability: implications of yeast mannoprotein additions prior to the bottling of red wine

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This Ph.D. project aims to investigate the impact of mannoproteins on winemaking, especially when added just before bottling, by studying their physicochemical and organoleptic effects, particularly on colour, mouthfeel and aromas.

Stabilità del vino: implicazioni delle aggiunte di mannoproteine del lievito prima dell'imbottigliamento del vino rosso

Questo progetto di dottorato si propone di indagare l'impatto delle mannoproteine nella vinificazione, soprattutto quando vengono aggiunte poco prima dell'imbottigliamento, studiando gli effetti fisico-chimici e organolettici, in particolare su colore, aroma e sapore dei vini.

Key words: mannoproteins; wine stability; physico-chemical and sensory parameters; by-products valorization.

1. Introduction

Yeast mannoproteins are highly glycosylated glycoproteins that contain about 80% D-mannose associated with D-glucose residues and N-acetylglucosamine, with 10-20% of proteins. They present a wide range of molecular weights that can typically vary from 5 to 400 kDa, but even up to 800 kDa. Their location is in the external layer of the yeast cell wall and are connected to a matrix of amorphous β-1,3 glucan by covalent bonds, making up to 35-40% of the cell wall. There are two moments in vinification when they are released: During alcoholic fermentation and after yeast autolysis by exogenous β-1,3-glucanase enzyme, being this last group similar but with less protein content (Rodrigues *et al*., 2012). Commercial preparations of yeast mannoprotein were first authorized for their addition in white wine to improve its tartaric and protein stability in the early 2000s, but then, its use quickly spread to red wines for other purposes than well-known chemical stabilization, starting to be attractive due to its apparent influence on technological and organoleptic effect on these wines. Within the already known enological properties of mannoproteins in wine production, the following can be named: inhibition of tartrate salt crystallization, reduction of protein haze, stimulation of malolactic fermentation, wine enrichment during autolysis of lees, interaction with flor wines, yeast flocculation, and autolysis in sparkling wines, adsorption of toxic ochratoxin; interaction with aromatic compounds, colour stabilization, reduction of astringency and increased body and mouthfeel sensations (Guadalupe and Ayestarán, 2008). The doctoral thesis project will explore the interaction between the physicochemical characteristics of mannoproteins and the wine matrix, which are not fully understood at the moment. The ultimate goal is to provide guidance on their selection and dosage before bottling, improving red wine quality.
This oral communication reports the main results of the following four activities directed to:
A1) Preliminary physicochemical and technological characterization of 5 commercial mannoproteins;
A2) Sensory analysis of two commercial qualities of Cabernet Sauvignon red wines of Chile, to which a standardized dose of mannose was applied prior to bottling for the same 5 commercial mannoproteins plus control;
A3) In-depth physicochemical and technological the same 5 commercial mannoproteins;
A4) selection of one mannoprotein and study 3 different doses applied to the same two commercial Cabernet Sauvignon wine qualities plus a control with measurements at 3 and 6 months of bottle aging.

2. Experimental Procedure

In this Ph.D. thesis project, the analyses and experiment were carried out as follows: A1) preliminary analysis and technological characteristics of 5 commercial mannoproteins specially indicated for its addition before bottling. A2) a standardized mannose dose of 5 of the same commercial mannoproteins was added to two commercial wine qualities, Blend and Premium, *Vitis vinifera* cv. Cabernet Sauvignon red wine of Chile in triplicate prior to bottling. A3) In parallel, a detailed analysis of the molecular weight distribution and monosaccharides composition of polysaccharides present were made in order to characterize these commercial mannoproteins and to evidence the possible presence of arabic gum. A4) The best commercial mannoprotein was

selected according to its physicochemical characteristics and organoleptic results in wine, using it for a second experiment in which doses of 3, 13.5 and 30g/HL were added before bottling to the same two commercial wine qualities, plus a control in triplicate. Analysis where measured at 3 and 6 months of bottle aging. Part of the results and analysis corresponding to the 3 months of aging in bottle will be presented in this report.

3. Materials and Methods

Physicochemical properties were measured, including total phenol and colour spectrophotometric indexes (280 nm, 420 nm, 520 nm, 620 nm, Hue and IC); Cielab colour space parameters (L*, a* and b*) calculated according to Ayala *et a*l. (1997); Total colour, colour due to free anthocyanins, due anthocyanins resistant to bisulfite and due to co-pigmentation (Levengood and Boulton 2004). The molecular weight of polysaccharides, monosaccharide concentration of them and total polysaccharide concentration were obtained by HPSEC-RID (Ayestarán *et al*., 2004), GC-MS (Guadalupe *et al.*, 2012), enzymatic analysis through of K-MANGL kit (Megazym ®) and spectrophotometric assay (Segarra *et al*., 1995). The filterability index was performed according to Meglioli *et al.*, (1983). The sensory analysis was conducted using Rate all that apply (R.A.T.A.) analysis, with 24 panelists, all of them enologist trained in sensorial analysis. The statistical methods used to analyse the data were multivariate analysis of variance (MANOVA) and Principal component analysis (PCA) through the software IBM SPSS Statistics version 25 and R-studio with R version 4.2.0.

4. Results and Discussion

4.1 Preliminary physicochemical and technological characterization of commercial mannoproteins

In A1, the 5 commercial mannoproteins used throughout the project were analysed for different physico-chemical parameters together with 3 other commercial mannoproteins in order to obtain a PCA grouping of them in terms of their technological aptitudes as the filterability index, the total polysaccharide content (and the concentration of mannose and glucose). This last information was used to determine a standardized dose of mannose in the following activities A2. The results of all these analyses can be seen in the PCA in **Figure 1**, in which the variables analyzed were: mannose:glucose ratio of the supernatant after three days of contact in model wine and centrifuged (Soluble Man:Gluc); mannose concentration of the supernatant after three days of contact in model wine and centrifuged (Soluble Man); total glucose directly from the commercial mannoprotein (Total Glu); sum of total glucose and mannose directly from the commercial mannoprotein (Total Glu+man); mannose concentration after three days of contact in model wine and centrifuged (Soluble Man); total polysaccharides concentration directly from the commercial mannoproteins (TPS), the percentage drop in the concentration of total polysaccharides directly added to model wine vs. after three days of contact and centrifuged (Precipitation TPS%). filterability index of commercial mannoproteins just added to model wine in a 15 g/HL dose (Filtration index 15g/HL).

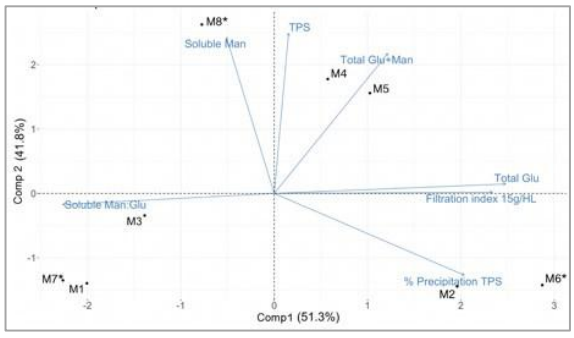

Figure 1 *PCA analysis using MANOVA significant variables ($p<0.05$), according to the Duncan post-hoc test, for the 5 commercial mannoproteins used throughout the project, plus 3 others commercial mannoproteins.*

4.2 Sensory analysis of commercial mannoproteins

Figure 2 presents the results of the PCA of the sensory variables that were significant in A2 for both qualities of wine, namely warmth, sweet, and smoothness. The plot shows that all the commercial mannoproteins tested were separated from the control at a normalized dose of mannose. Among the commercial mannoproteins, MP5 was highlighted as the most different and was the only one that exhibited significant differences in all three sensory parameters compared to the control. The normalization based on mannose content allowed an effective comparison among commercial mannoproteins

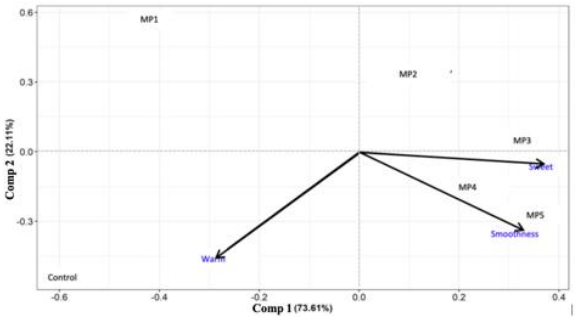

Figure 2 *PCA plot of RATA (Rate All that Apply) significant sensorial variables (p<0,05), according to HSD Tukey post-hoc test.*

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4.3 Analysis of molecular weight distribution and monosaccharides of polysaccharides of commercial mannoproteins

In parallel, in activity A3, analyses were performed on the same 5 mannoproteins to determine their molecular weight distribution and the total concentration of polysaccharides, monosaccharides, and proteins. The results are presented in **Figure 3 A-F**. The analysis revealed that certain mannoproteins had a mixture of medium (~40-50 kDa) and high (~80-150 kDa) molecular weights, while others contained only medium or low (~25 kDa) molecular weights. An important concentration of molecular weights <5kDa (Oligosaccharides) was also found for some of the mannoproteins. The above could explain the different results obtained in sensorial analysis, considering that a standardized dose of mannose was applied for both qualities of wine. Another important finding was the elevated concentration of monosaccharides structurally associated with arabic gum in mannoprotein MP3, a regulated additive in wines exported to China, a crucial market for Chilean wines.

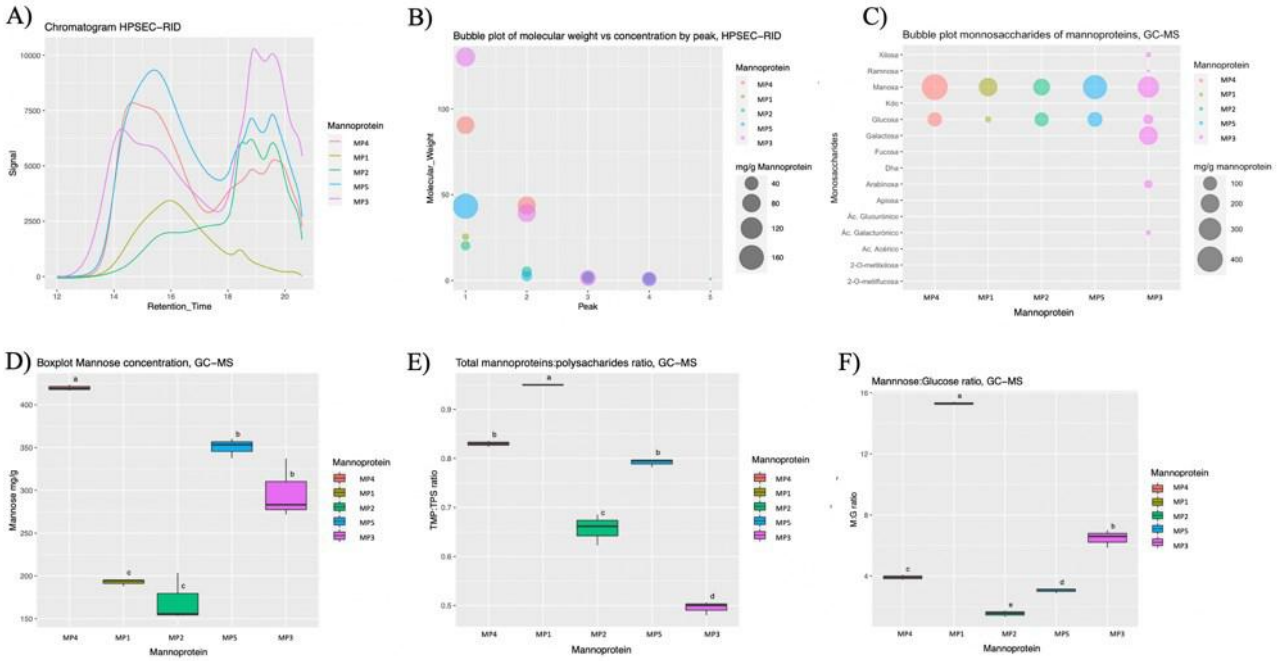

Figure 3 *Polysaccharide and monosaccharide analysis of commercial mannoproteins by HPSEC-RID: A) Chromatogram, B) molecular weight distribution, and GS-MS: C) monosaccharides concentration, D) mannose concentration, E) Total mannoprotein to polysaccharides ratio, F) mannose to glucose ratio.*

4.4 Cielab analysis, MANOVA and PCA of physicochemical and volatile compound analysis

The results of physicochemical analysis for 3 months of bottle aging of A4 are shown in the PCA of **Figures 4 a-d**, revealing a significant increase in Hue for both wines with increasing doses of selected mannoprotein MP5 when compared to the control. In addition, Blend wine quality distances itself from the control at any dosage, while Premium wine does it only at a dosage of 13.5 g/HL onwards. On the other hand, it was observed that there was a very different and opposite evolution of colour for both wine qualities, specifically in terms of % of total colour (WC) due to monomeric anthocyanins (MAC%) and % of total colour due to bisulfite stable anthocyanins (BSA%), but also in Cielab colour space L* parameter. Suggesting that different polymerization and precipitation processes took place depending on the different wine matrices and the increasing dose of

mannoprotein before bottling.

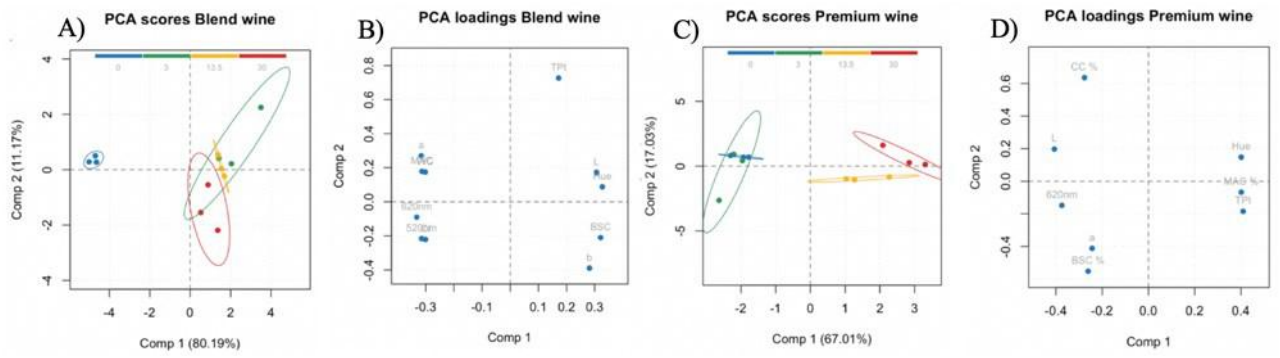

Figure 4 Scores and loadings of *PCA analysis using physicochemical MANOVA significant variables (p<0.05), according to the Duncan post-hoc test, for each wine quality. A-D) score and loading plots for Blend and Premium quality wines. Doses: 0 (blue), 3 (green), 13.5 (yellow), and 30 (red) g/HL.*

In this sense, **Figure 5** illustrates how the dosage has a greater impact on the total colour of Blend wines, particularly at doses exceeding 3 g/HL, while having no significant effect on the total colour of Premium wines. However, it is worth noting that in the case of the last, the perceived darkness of the colour, as indicated by Cielab, may exhibit a slight increase at higher doses due to a marginal but significant reduction in L*.

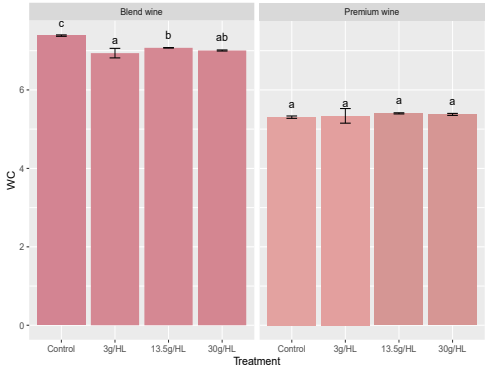

Figure 5 *Total colour according to Boulton for each dosage and wine quality. Different letters on the same line indicate statistically significant differences (p < 0.05) according to Duncan's post hoc test. The different observed colours of each bar are obtained through L*, a* and b* parameters.*

The last can be corroborated by the **Table 1** where it is shown that only in the case of Premium in doses from 13.5 g/HL and higher lead to an observed colour difference with the control, calculated according to García-Marino et al., (2013), where values above 2.7 CIELAB units indicate colour differences that are detectable to the human eye, that in this case, are explained mainly by a lower L* Cielab parameter.

Table 1 *Colour differences (ΔE*ab) matrix between two colour points in the CIELAB*

	Blend wine				Premium wine			
	A	**B**	**C**	**D**	**A**	**B**	**C**	**D**
A) Control	0.0	1.9	1.9	1.7	0.0	1.6	**4.7**	**4.6**
B) 3 g/HL	1.9	0.0	0.1	0.6	1.6	0.0	**4.4**	**4.1**
C) 13. 5g/HL	1.9	0.1	0.0	0.6	**4.7**	**4.4**	0.0	0.4
D) 30 g/HL	1.7	0.6	0.6	0.0	**4.6**	**4.1**	0.4	0.0

On the other hand, the results of PCA shown in **Figure 6 a-d** show that the analysis of volatile compounds also presents a different response to mannoprotein dose, depending on the quality of the wine. In the case of the Blend quality, 3 g/HL showed a higher concentration of total volatile compounds, while 30 g/HL was the lowest. In the case of Premium, a grouping similar to that of the physicochemical PCA was observed, with a tendency to increase the concentration of volatile compounds at higher doses and obtain the highest total concentration of volatile compounds at the dose of 13.5 g/HL.

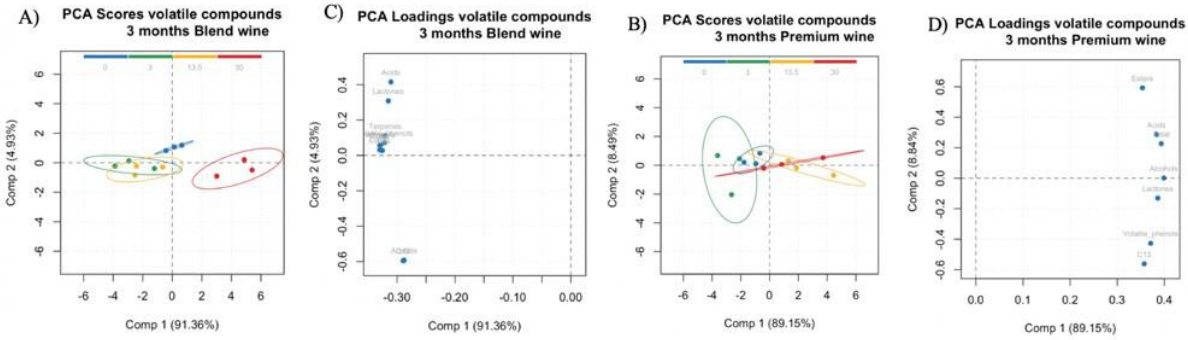

Figure 6 Scores and loadings of *PCA using volatile compounds MANOVA significant variables (p<0.05), according to the Duncan post-hoc test, for each wine quality. A-D) score and loading plots for Blend and Premium quality wines. Doses: 0 (blue), 3 (green), 13.5 (yellow), and 30 (red) g/HL.*

4.5 Efficiency of mannoprotein addition according to dosage and wine quality

Finally, as seen in **Figure 7**, there is a clear tendency to decrease the remaining mannoprotein of wines depending on the dose applied. This may mean that it is not economically worthwhile to add more than 13.5 g/HL for both qualities of wine.

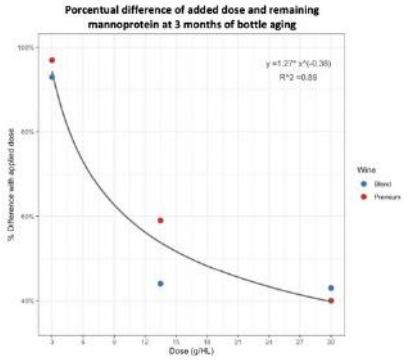

Figure 7 *Applied dosage vs. remaining concentration (%) after 3 months of bottle aging.*

5.Conclusions and Future Perspectives

The research found that, after 3 months of bottle aging, increasing doses of mannoprotein in the wine can significantly increase the Hue and modify the colour evolution and the concentration of volatile compounds differently depending on the wine matrix and the dosage used. Based on the information obtained, it would not be advisable to use more than 3 g/HL before bottling in the case of Blend quality and 13.5 g/HL in the case of Premium quality, which is consistent with commercial recommendations dosages for this commercial mannoprotein and these specific Cabernet Sauvignon Chilean qualities of wines. In addition to the above results, other analyses are being performed as the determination of the total concentration of anthocyanins, phenols, polysaccharides and its monosaccharides, together with sensory analysis for the first three months. On the other hand, further analysis are in progress on 6 months of bottle aging to better understand which and why a given dosage is best suited to each wine matrix in terms of physico-chemical and sensory characteristics. Understanding the differences observed will encourage the use of this natural by-product of winemaking among enologists seeking to improve red wine quality in a sustainable manner.

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Application of functional molecules recovered from bergamot by-products: development and improvement of food systems

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In the last years, bergamot (*Citrus bergamia Risso*) has shown great interest due to its beneficial effect on human health due to the high content of phenolic compounds. The aim of the work is to valorise bergamot pomace recovering this fraction through a selected extraction method (among conventional, ultrasound and microwave extraction) and application in food systems. The use of the obtained liquid extract rich in phenols, was tested in an edible coating to study its effect strawberries shelf life. Moreover, the extract was microencapsulated investigating its impact during storage of enriched lipophilic and hydrophilic food matrices.

Applicazione di molecole funzionali recuperate da pastazzo di bergamotto: sviluppo e miglioramento di sistemi alimentari

Negli ultimi anni, il bergamotto (*Citrus bergamia Risso*) ha riscontrato grande interesse per i suoi effetti benefici sulla salute umana grazie all'elevato contenuto di composti fenolici. Lo scopo del lavoro è quello di valorizzare il pastazzo di bergamotto recuperando questa frazione attraverso un metodo di estrazione selezionato (tra estrazione convenzionale, ultrasuoni e microonde) e l'applicazione in sistemi alimentari. L'uso dell'estratto liquido ottenuto, ricco di fenoli, è stato testato in un rivestimento edibile per studiare il suo effetto sulla shelf life delle fragole. Inoltre, l'estratto è stato microincapsulato per studiarne l'impatto durante la conservazione di matrici alimentari lipofiliche e idrofiliche arricchite.

Keywords: Bergamot by-product; phenolic extraction; edible coating; microencapsulate; functional foods

1. Introduction

One of the most important citrus fruits grown in Calabria is Bergamot, which represents a valuable source of active molecules that contribute to antioxidant, anti-inflammatory, and cholesterol reduction capacities (Da Pozzo *et al.*, 2018; Schwingshackl *et al.*, 2020). After its industry processing, waste is an industrial problem to manage. Nevertheless, the high amount of antioxidants, especially flavonoids, allow to consider it as source of natural additives.
In accordance with the PhD thesis project, this oral communication reports the main results of the following activities:
A1) Selection of the best extraction to recovery bioactive compounds from bergamot pomace;
A2) Application of the best extract (AE) to edible coatings for strawberries' shelf life extension;
A3) Effect of microencapsulated AE in lipophilic and hydrophilic food systems.

2. Materials and Methods

2.1 Extraction of antioxidant compounds

Bergamot pomace (BP) represented by skins, pulp and seeds, was subjected to dehydration (at 50°C) to reduce the moisture content (up to 12%) and powdered by mean of a laboratory mill to facilitate the extraction process. In order to obtain an extract with high antioxidant power, different techniques were carried out. Three extraction methods were tested: conventional maceration (C), ultrasound (UA) and microwave (MA) assisted extraction; water and ethanol/water mixture (50:50, v/v) were considered as solvents (food grade). For C and UA methods the extraction was carried out with different combination of temperature (25 and 70 °C) and time (30 and 60 min), while for MA the extraction time was different (5 and 15 min). The best extraction conditions were evaluated referring to the main physicochemical characteristics and antioxidant activity and constituents, such as total phenols and flavonoids, total antioxidant activity, individual flavonoids and limonoids (UHPLC-DAD), as reported by Gattuso *et al.* (2023). The obtained extracts were characterized based on dry weight of BP (dw: 17%). Later, the best selected extract obtained by conventional maceration at 70°C for 30 minutes in an hydroalcoholic mixture (AE), was prepared using 200 g of BP and 800 mL of solvent and was applied in the formulation of an edible coating solutions and for the preparation of the microencapsulated.

2.2 Edible coating formulation and application on strawberries

The coating was prepared following the method reported by Tahir *et al.* (2018) using a concentration of arabic

gum of 2%. AE and BHT solutions were added and heated at 40 °C for one hour under continuous stirring. Subsequently, 1% glycerol (v/w) was added as plasticizer to improve the strength and flexibility of the coating solutions. The concentrations of AE and BHT added to the coatings were: 100 ppm of BHT (sample B); 1% AE (sample C); 2.5% AE (sample D); 5% AE (sample E). Additionally, a control sample (A) was prepared. Strawberries were dipped in the different coating solution for 3 min and the excess of the coating was drained and air-dried (under UV and at room temperature to prevent environmental contamination). The fruit samples were packaged into hinged food containers (PET) in normal atmosphere and stored at 4 °C. Shelf-life study was conducted monitoring changes in strawberries (3, 7, 10, and 14 days).

2.3 Encapsulation of AE and application in sunflower oil and apple juice

For the preparation of the microencapsulate, maltodextrin at concentrations of 20% was added and the sample was lyophilised as reported by Ballesteros *et al.* (2017). After, 2% of microencapsulate (MD20) was used to enrich apple juice (EJ) and sunflower oil (EO), this concentration was choice after different tests to verify the best response in food. For each product three darkened containers for each monitoring time were prepared and stored at 25°C until further analysis.

2.4 Food products characterization

Organic acids, microbiological counts, texture in strawberries and characterization of the phenolic profile of MD20, EJ and EO were carried out as reported by De Bruno *et al.* (2023). To investigate the effect of MD20 in EO during storage time an oxidative stress test was conducted by mean of OXITEST system (Imeneo *et al.*, 2021).

2.5 Statistical analysis

Results of the present study were expressed as mean ± SD of three measurements (n = 3), except for strawberries' firmness in which ten measurements were conducted. Appropriate test statistics, One-way ANOVA with Tukey's post-hoc test, and t-test were at $p < 0.05$ were performed by SPSS Software (Version 15.0, SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1 Characterization of the best selected extract

The best selected extract (AE) was obtained by conventional maceration at 70°C for 30 minutes, using as solvent a hydroalcoholic mixture (H_2O/EtOH 50:50 v/v). Ethanol is environmentally friendly and allows to obtain a good extraction of polyphenols (Gil-Martín *et al.*, 2022). The extraction procedures provide to convert citrus waste in a source of value-added products to use in functional foods as widely demonstrated in the literature (Andrade *et al.*, 2023). As reported in Table 1, the selected extract exhibited a phenolic content of 26.30 mg gallic acid equivalent (GAE) g^{-1} dw and 6.18 mg catechin equivalent (CE) g^{-1} dw. The total antioxidant activity level was investigated with DPPH and ABTS assays, in which it showed values of 1.6 and 16.93 mmol Trolox equivalent (TE) g^{-1} dw, respectively. The main individual flavonoids and limonoids analysed by chromatographic analysis (UHPLC-DAD) were reported in Table 1. Neoeriocitrin, naringin, neohesperidin, brutieridin and melitidin were the main flavonoids detected in BP, as also reported by other authors (Di Donna *et al.*, 2020; Gorinstein *et al.*, 2001).

The best selected method was chosen due to its high content of bioactive compounds. For TPC, AE and the extract obtained with the same method prolonged for 60 minutes, showed similar content (26.30 and 26.06 mg GAE g^{-1} dw, respectively), although UA also led to a good recovery of TPC (about 23.64 mg GAE g^{-1} dw extract at 25°C for 30 min with H_2O/EtOH). Compared to the other types of extraction tested, AE showed 60% higher extraction than MAE performed at 25°C for 5 min with H_2O/EtOH. In total antioxidant activity the ABTS assay was also considered in the selection of the method. The assay displayed values that ranged between 3.22 and 16.93 mM TE g^{-1} dw. The best extraction in terms of total antioxidant activity was obtained by applying the hydroalcoholic solution (H_2O/EtOH) as extraction solvent and assisted by C at 25°C 60 min and in AE confirming the highest recovery of bioactive compounds in the last one. The choice was established also comparing the concentration of the major flavonoids detected. The main abundant flavonoids (neoeriocitrin, naringin, neohesperidin and brutieridin) were always found in the highest concentrations in AE and in the extract obtained for C at 70°C for 60 min with H_2O/EtOH mixture, as was the case for TPC. Also, for the extraction of limonoids, AE showed the highest quantity recovered. In the selected extract, limonin was higher almost 20% compared to all extractions, reaching more than 90%. For nomilin lower differences were found in the extracts resulting by the use of hydroalcoholic mixture and with UA for a time of 30min and 25°C, and the extract in C at 25°C for 60 min, respectively 13.10% and 14.25% but more than 80% in others. In conclusion, data highlighted

Table 1 *Antioxidant characterization of AE.*

TPC (mg GAE g^{-1} dw)	26.30±0.09
TFC (mg CE g^{-1} dw)	6.18 ± 0.12
DPPH (mm TE g^{-1} dw)	1.6± 0.17
ABTS (mm TE g^{-1} dw)	16.93± 0.59
Eriocitrin*	0.25± 0.00
Neoeriocitrin*	13.95± 0.07
Naringin*	12.47± 0.04
Neohesperidin*	7.35± 0.06
Melitidin*	2.29± 0.01
Brutieridin*	5.67± 0.04
Limonin*	7.80± 0.03
Nomilin*	8.04± 0.06

*mg g^{-1} dw

that for the parameter considered, the conventional maceration extraction system produced the greatest extractability of bioactive compounds. As observed previously in other food matrices, the hydroalcoholic solvent is the best choice to obtain the maximum yield of antioxidant compounds, when combined to 70°C of temperature (Chemat *et al.*, 2017).

3.2 Effect of edible coating on strawberries

Generally, minimally treated fresh fruit have a short shelf-life (4–7 days), which is very important to preserve the freshness of the fruit and avoid excessive losses due to the reduction in their quality. In Table 2, the microbiological results are reported. The Total Bacterial Count (TBC), yeasts and molds were revealed already since the 1st day of storage in samples A (control) and B, while the other samples did not show any contamination. The use of AE in edible coatings provide advantages to preserve their high sensibility to microbial decay. The obtained microbiological values fall within the acceptable limits of a maximum aerobic plate count of 5×10^7 cfu/g at the end of shelf life for different fresh-cut vegetables as reported by Fan and Song (2008). During the storage, increases in all analysed microbiological parameters were observed, particularly at 14 days of storage. This grow could be also related to humidity conditions developed inside the containers. All the samples showed an increment of yeasts and molds during the storage time. After 14 days, for yeasts the highest concentration was found in A (7.0 Log10 CFU g^{-1}), while for molds, in A and B samples. These values showed that the application of edible coatings is useful to improve and extend the quality of strawberries.

Table 2 *The microbiological counts of minimally treated strawberries (Log10 CFU g^{-1}).*

	TBC				***Yeasts***				***Molds***			
	1st	***7th***	**14th**	**Sign.**	**1st**	7th	14th	**Sign.**	1st	7th	14th	**Sign.**
A	1.8 aC	2.9 aB	3.4 bA	**	1.1 aC	4.9 bB	7.0 aA	**	3.0 aB	3.5 aB	5.3 aA	**
B	1.0 bC	2.0 bB	4.1 aA	**	1.1 aB	6.1 aA	6.4 bA	**	2.1 bB	2.1 bB	5.3 aA	**
C	0 cC	2.5 abB	3.7 abA	**	0 bB	5.8 aA	5.2 cA	**	0 cB	0 cB	4.4 bA	**
D	0 cB	2.9 aA	2.4 cA	**	0 bB	4.9 bA	6.4 bA	**	0 cC	2.1 bB	4.8 abA	**
E	0 cC	1.3 cB	3.1 bA	**	0 bC	1.5 dB	4.7 cA	**	0 cB	0 cB	3.2 cA	**
Sign.	**	**	**		**	**	**		**	**	**	

The fruit firmness was analysed on the coated samples during the storage time (Table 3), because it represents one of the essential parameters to determine fruit quality. The softening is a natural physiological effect of fruit ripening with cell wall changes and the dissolution of the middle lamella, which in turn causes loss of cell-to-cell adhesion (Chen *et al.*, 2011; Villarreal *et al.*, 2016). At time 0, the firmness of the fruit was 10.1 N, while after seven storage days, a decreased was observed, both in the uncoated and coated samples with lowest value found in the control sample (3.21 N), similar to sample B (3.62 N). The highest firmness values were recorded in sample D at 6.32 N. Additionally, after 14 days of cold storage, samples A and B showed the lowest firmness values, 1.54 N and 3.22 N, respectively. The results were in accordance with those of Tahir *et al.* (2019), in which the retention of flesh firmness of blueberries was achieved by the combined effect of African baobab pulp extract and arabic gum; while Kahramanoğlu *et al.* (2022) reported that uncoated strawberries over time showed a significant decrease in firmness than in those coated. As also demonstrated by statistical analysis, at the end of the storage time, D and E samples showed the highest values of firmness among the samples.

Table 3 *Firmness in strawberries samples during storage.*

	FIRMNESS (N)					
	1st	3rd	7th	10th	14th	Sign.
A	10.10A	6.32cdB	3.24cC	2.69dD	1.54cE	**
B	10.10A	7.01cB	3.62cC	3.58cC	3.22bD	**
C	10.10A	8.63aB	4.31bC	4.03bCD	3.85abD	**
D	10.10A	9.61aB	6.32aC	5.95aC	4.18aD	**
E	10.10A	8.02bB	4.43bC	4.28bD	4.08aD	**
Sign.	n.s.	**	**	**	**	

Ascorbic acid (AA), or vitamin C, is one of the major components of strawberries, and its content is an indicator of quality relevant to define freshness of fruits (Cordenunsi *et al.*, 2023). Many authors have reported that decrease of AA during storage is caused by its oxidation (Atress *et al.*, 2010) and by respiration rate of the fruit (García *et al.*, 1996). The use of a coating promotes protection against both effects. As is possible to see in Table 4, the results obtained confirm this effect. The control sample (A) showed significant variation of the AA content values during the storage period ($p < 0.01$), with the lowest value being shown at 14 days. The initial AA content was 33.01 mg 100 g^{-1}, and after seven days it decreased to 28.35 mg 100 g^{-1}, while at 14 days it was 27.02 mg 100 g^{-1} (the lowest detected value) After 14 days, also the sample B showed a low content (29.07 mg 100 g^{-1}), while the samples coated with AE solution showed the highest values. Citric acid is the predominant organic acid in strawberries. The data detected during this experimentation process are reported in Table 4. There were great losses (highly significant, $p < 0.01$) of this organic acid, particularly in the control sample (A), from 692.5 mg 100 g^{-1} at the beginning to 402.9 mg 100 g^{-1} at the end of the shelf life (14 days). Regarding the trend shown during the storage period, the CA contents varied significantly only in two samples, A and B ($p < 0.01$); all other

samples meanwhile samples treated with AE showed no significant differences ($p > 0.05$). All coatings enriched with AE highlighted the good stability of this acid over time.

Table 4 *Ascorbic and citric acid content in strawberry samples (mg 100g^{-1}).*

Ascorbic Acid	***A***	***B***	***C***	***D***	***E***	***Sign.***
1st	33.0 ± 0.5 ^{a}	33.0 ± 0.5 ^{a}	33.0 ± 0.5 ^{a}	33.0 ± 0.5 ^{a}	33.0 ± 0.5 ^{a}	n.s.
7th	28.4 ± 0.1 ^{bC}	27.3 ± 0.4 ^{bC}	32.6 ± 0.1 ^{aA}	29.6 ± 0.8 ^{bBC}	31.4 ± 1.02 ^{abAB}	**
14th	27.0 ± 0.2 ^{cB}	29.1 ± 0.3 ^{bB}	30.3 ± 0.1 ^{bA}	31.5 ± 1.4 ^{abA}	29.8 ± 0.5 ^{bA}	*
Sign.	**	**	**	*	*	
Citric Acid	***A***	***B***	***C***	***D***	***E***	***Sign.***
1st	692.5 ± 26.5 ^{a}	692.5 ± 26.5 ^{a}	692.5 ± 26.5	692.5 ± 26.5	692.5 ± 26.5	n.s.
7th	604.1 ± 42.0 ^{aB}	669.1 ± 2.2 ^{aB}	720.5 ± 10.2 ^{A}	745.7 ± 2.1 ^{A}	698.0 ± 12.3 ^{A}	*
14th	402.9 ± 3.3 ^{bC}	583.5 ± 56.0 ^{bB}	711.1 ± 20.3 ^{A}	727.5 ± 32.1 ^{A}	676.8 ± 17.9 ^{AB}	*
Sign.	**	**	n.s.	n.s.	n.s.	

3.3 Evaluation of microencapsulate and enriched food products (EJ and EO)

In accordance with the above, in Table 5 are shown the main flavonoids identified in MD20. Flavonoid concentrations in enriched juice during monitoring times displayed statistical differences (Table 6) for all of them. In EJ storage at 25°C the major flavonoids, neoeriocitrin, naringin and neohesperidin followed the same trend with an increment after 90 days. The other compounds followed a decrease at the 90th day of monitoring. Specifically, eriocitrin, narirutin and brutieridin exhibited a stable trend after 45days with values comparable to T0, and a significative ($p<0.01$) reduction at the last monitoring time. Melitidin presented a small decrement after 45 days passing from 19.94 mg L^{-1} (T0) to 17.18 L^{-1} (T45) reaching 5.37 mg L^{-1} at T90. These different trends could be due to a gradual and different release of single compounds. However, the overall content increased over time, showing a gradually release of phenolic compounds. This effect was also observed by Wyspiańska *et al.* (2019), who found a gradually increasing of isoflavonones after a degradation over time of maltodextrin capsules with a release of phenolic molecules in the solution.

Table 5 *Flavonoids composition of MD20.*

MD20 (mg g^{-1})	
*Eriocitrin **(1)***	0.2 ± 0
*Neoeriocitrin **(2)***	7.32 ± 0.09
*Narirutin **(3)***	0.11 ± 0.01
*Naringin **(4)***	8.55 ± 0.1
*Neohesperidin **(5)***	4.75 ± 0.19
*Melitidin **(6)***	1.27 ± 0.08
*Brutieridin **(7)***	3.2 ± 0.11

Table 6 *Flavonoids Flavonoid composition of Enriched Juice.*

	EJ (mg L^{-1})			
	T0	**T45**	**T90**	**Sign.**
1	2.29 ± 0.1^{a}	2.31 ± 0.1^{a}	1.23 ± 0.29^{b}	*
2	88.18 ± 1.45^{b}	90.41 ± 2.08^{b}	107.16 ± 1.99^{a}	**
3	0.85 ± 0.04^{a}	0.87 ± 0.03^{a}	0.11 ± 0.01^{b}	**
4	104.38 ± 1.47^{b}	111.54 ± 3.02^{b}	129.81 ± 0.63^{a}	**
5	57.44 ± 1.21^{b}	63.18 ± 1.93^{b}	94.41 ± 0.71^{a}	**
6	19.94 ± 0.56^{a}	17.18 ± 0.2^{b}	5.37 ± 0.31^{c}	**
7	41.12 ± 0.42^{a}	41.23 ± 1.79^{a}	4.68 ± 0.08^{b}	**

The microencapsulate in sunflower oil showed a different behaviour that in juice as reported in Table 7. Except for melitidin (6), differences of flavonoids content where found during the storage of EO. Eriocitrin (1), neoeriocitrin (2) and brutieridin (7) evidenced the same trend with a maximum level at 45th day and a subsequently slightly decrease ($p<0.05$). Narirutin (3) increased in the second monitoring time to decrease below the initial value at the end. High statistical differences ($p<0.01$) were also found in naringin (4) content which revealed an initial value of 47.1 mg L^{-1}, and higher values at T45 (58.89 mg L^{-1}) and T90 (56.26 mg L^{-1}). Moreover, neohesperidin (5), after an initial increase maintained constant concentration. The gradually release of encapsulated antioxidants in oil phase was also observed by Mohammadi *et al.* (2016). The different behaviour of the microencapsulate in EJ and EO could be due to the hydrophilic nature of maltodextrin, which is hydrophilic and high soluble in water, permitting an easy release of compounds from the capsules (Hermanto *et al.*, 2016). This could also explain a faster solubilization of the microencapsulate in EJ founding a higher amount of phenolic compounds just from the beginning of the experimentation. In edible oil the oxidation control is a parameter that defines its quality and safety for human health. It depends on several factors intrinsic and extrinsic, and the enrichment with natural antioxidants is a good strategy for its preservation (Fadda *et al.*, 2022). In this study is reported the oxidative stability analysed with OXITEST, which was expressed as induction period (IP). IP represents the time needed to get to the point where oxidation begins. It was analysed in order to evaluate the resistance of fat matrix to oxidation (De Bruno *et al.*, 2021). As expected, the presence of phenolic compounds added with MD20, resulted in a significantly grown in IP (Figure 1) after 45 days at the storage temperature of 25°C, reaching 14:12 (h:m) starting from 13:16 (h:m) with a consequently enhancement due to the antioxidant effect of bioactive compounds as observed by De Bruno *et al.* (2022). After 90 days, the IP of EO showed lower value compared to IP of T0 due to different mechanism reactions given to the natural irreversible oxidation combined with the storage temperature.

Table 7 *Changes in flavonoids in Enriched Oil during storage.*

	EO (mg L^{-1})			
	T0	**T45**	**T90**	**Sign.**
1	1.03 ± 0.02^{b}	1.27 ± 0.03^{a}	1.13 ± 0.05^{ab}	*
2	37.77 ± 1.25^{b}	46.71 ± 2.04^{a}	43.31 ± 1.13^{ab}	*
3	0.97 ± 0.04^{b}	1.28 ± 0.08^{a}	0.39 ± 0.02^{c}	**
4	47.1 ± 0.66^{c}	58.89 ± 0.4^{a}	56.26 ± 0.65^{b}	**
5	25.37 ± 0.62^{b}	32.4 ± 1.61^{a}	30.32 ± 0.14^{a}	*
6	8.84 ± 0.03	10.42 ± 0	9.53 ± 1.41	n.s.
7	18.01 ± 0.79^{b}	22.87 ± 0.77^{a}	20.4 ± 0.48^{ab}	*

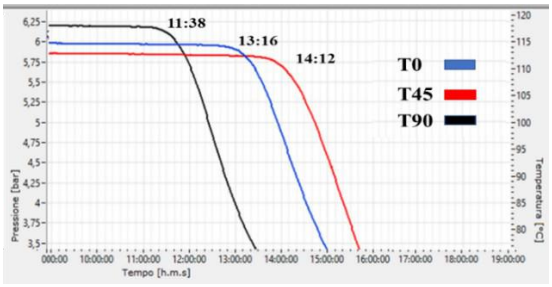

Figure 1 *Oxidation curves at T0, T45 and T90 of EO.*

4. Conclusions and Future Perspectives

These studies have proposed strategies for the valorisation of bergamot pomace, highlighting its high content of antioxidant compounds recoverable through food grade extractions, and its use applicable to the food industry in the formulation of edible coating, and the enrichment of juices and oils. This study also made possible to highlight the natural preservative effect to extend shelf life of strawberries, and on the other hand to supply antioxidant compounds to poor foods such as apple juice or seed oil, improving, in the case of oil also the oxidative stability. Overall, the use of this by-product has made possible to obtain foods with high added value, contributing to the reduction of citrus waste. The results obtained are very promising and the future research could focus on the purification of the extract evaluating the effect of the single compounds, and to set-up in vivo trials to confirm their functionality.

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The role of root exudates in promoting beneficial interactions and rhizoremediation potential of polychlorinated biphenyls (PCBs)-degrading bacteria

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This PhD thesis investigates the crosstalk between plants and soil bacteria mediated by root exudates, in the context of soil contamination by Persistent Organic Pollutants (POPs) like polychlorinated biphenyls (PCBs). The work addresses the role that flavonoids, plant secondary metabolites, play in influencing rhizocompetence traits, necessary for root colonization, in a model PCB-degrading bacterial strain. The results obtained contribute to improve the knowledge of plant beneficial interaction with PCB degrading bacteria, potentially improving the effectiveness of soil phyto-rhizoremediation strategies for PCBs removal.

Ruolo svolto dagli essudati radicali nella promozione delle interazioni benefiche e nella capacità di biorisanamento di batteri degradatori di policlorobifenili (PCB)

Questa tesi di dottorato studia le interazioni pianta-microrganismo mediate dagli essudati radicali, in particolare nel contesto della contaminazione del suolo da parte di inquinanti organici persistenti come i policlorobifenili (PCB). Il lavoro si concentra sul ruolo che i flavonoidi, metaboliti secondari delle piante, svolgono nell'influenzare i tratti di rizocompetenza in un ceppo batterico modello che degrada i PCB, necessari per la colonizzazione delle radici. I risultati ottenuti contribuiscono a migliorare la conoscenza delle interazioni benefiche delle piante con i batteri degradatori, potenzialmente migliorando l'efficacia delle strategie di fitorisanamento del suolo per la rimozione dei PCB.

Key words: Root exudates; flavonoids; root colonization; plant holobiont; PCBs; phyto-rhizoremediation.

1. Introduction

In line with the objectives of the PhD project previously illustrated (Ghitti, 2021), this oral communication reports the main results obtained during the doctorate studies, aimed at:
A1) The critical analysis of scientific literature on the role of root-exuded secondary metabolites in shaping bacterial communities with degradative potential in contaminated soils, with a special focus on flavonoids;
A2) Investigating the different root exudation profile of the model plant *A. thaliana* in presence and absence of PCB stress and its impact on PCB-degrading bacteria activity and metabolism;
A3) Investigating the role of specific flavonoids on the model PCB-degrading bacterium *Paraburkholderia xenovorans* LB400, focusing on the improvement of traits necessary for efficient root colonization;
A4) Generation of fluorescent-tagged bacteria to analyze the root colonization profile under PCB stress.

2. "Cry-for-help" mediated by root exudates in contaminated soils

Plants live in close association with a multitude of microorganisms coevolving together as a unique meta-organism defined as the plant holobiont. The crosstalk between plant and microorganisms, particularly those colonizing the rhizosphere and the endosphere, is carried out through root-exuded primary and secondary metabolites that act as essential chemical signals to maintain the health status of the holobiont (Vandenkoornhuyse *et al.*, 2015). Root chemistry shapes a rhizospheric microbial community that can provide benefits for the holobiont: it was hypotesized that, when exposed to stress due to the presence of phytopathogens, the plant enacts a 'cry-for-help' by exuding specific metabolites necessary for the recruitment of beneficial microorganisms to counteract the attack (Rolfe *et al.* 2019). The same mechanism was hypothesized as part of the adaptation strategy for plants exposed to abiotic stresses like the presence of phytotoxic xenobiotic contaminants, such as polychlorinated biphenyls (PCBs), in soil. Since plants often lack the catabolic enzymes needed for complete degradation of PCBs, they can resort to the exudation of specific metabolites to recruit PCB-degrading bacteria that could degrade recalcitrant contaminants and decrease their phytotoxicity (Rolli *et al.* 2021). Flavonoids are root-exuded secondary metabolites that are among the most promising molecules acting as inducers or co-metabolites to trigger the expression of the catabolic genes for biphenyl aerobic degradation, encompassed by the *bph* operon (Pham *et al.* 2015; Zubrova *et al.* 2021). This mechanism could be exploited to improve the degradation and removal of PCBs from the environment through phyto-

rhizoremediation.

3. Plant secondary metabolites influence bacterial root colonization

Root exudates are necessary to establish close and stable associations with plant growth promoting bacteria, that contribute to the holobiont fitness by alleviating nutritional shortages, producing phytohormones necessary for plant development or by acting as biocontrol agents. Among these metabolites, flavonoids are well known for their involvement as chemical prompts in initiating rhizobia-legume symbioses but were also studied for their role in interacting with other soil microorganisms and influencing bacterial root colonization. For instance, some beneficial soil bacteria can metabolize plant flavonoids and use them as carbon sources (Pillai and Swarup 2002) while for others, flavonoids are involved in the modulation of rhizocompetence traits like bacterial motility (Yu *et al.* 2020) or biofilm formation (He *et al.* 2022) for the stable colonization of root surfaces. Although emerging evidence about flavonoids' role in plant interaction with non-rhizobia microorganisms was observed, these is still a lack of knowledge about the mechanisms underlying these relationships.

4. Materials and Methods

4.1 *In vitro* assays to test rhizocompetence traits

Bacterial metabolism and features involved in root colonization were investigated on three PCB-degrading bacterial strains through adapted *in vitro* assays previously reported in literature using pure metabolites. Bacterial growth stimulation by flavonoids was tested as reported by Huang *et al.* (2019) by adding µM concentrations of pure flavonoids to a diluted growth medium. Swimming motility was assayed in plates containing semi-solid 0.25% agar medium supplemented with flavonoids, as well as chemotaxis motility, tested as reported by Reyes-Darias *et al.* (2015) using a gradient plate medium assay. Biofilm formation was tested in 96 well plates using the crystal violet staining method to test bacterial adhesion (Yoshioka and Newell 2016).

4.2 Generation of fluorescence-labelled bacteria

Two fluorescent bacterial strains were engineered and used to observe *Arabidopsis* root colonization pattern. *Paraburkholderia xenovorans* LB400 was tagged chromosomally with the red fluorescent protein *mScarlet,* expressed under a constitutive promoter, through filter-mating conjugation, using as donor the *E. coli* S17-1 helper strain carrying the pMRE-Tn5-145 transposon delivery plasmid (Schlechter *et al*., 2018). The strain *Pseudomonas alcaliphila* JAB1 was engineered with the plasmid pUCP18-CmR-IR_GntR-egfp, kindly donated by prof. Ondřej Uhlík (UCT Prague), to obtain a biosensor. The plasmid harbored an inducible eGFP gene putatively regulated as the *bphA* gene, that encodes for the protein necessary for the first step of the aerobic biphenyl degradation pathway. Furthermore, *P. alcaliphila* JAB1 biosensor was tagged with a constitutive *mScarlet* by conjugation to observe the bacterial root colonization profile.

5. Results and Discussion

5.1 PCB stress induces a shift in *Arabidopsis* root exudation profile

The root exudates of *Arabidopsis thaliana* (ecotype Col-0) were collected 2 days after the induction of stress with 70 µM PCB and analyzed through metabolomics. The abundance of 62 compounds was found statistically different in PCB-treated root exudates compared to the untreated control and five of these metabolites were further identified. The coumarin scopoletin and N-(2-hydroxyethyl)-β-alanine decreased their relative abundance in presence of PCB, while hypoxanthine and two dipeptides (L-seryl-L-phenylalanine and L-arginil-L-valine) were exuded in higher amount. Reduced exudation of scopoletin under PCB stress might be due to the antimicrobial activity often exerted by coumarins (Voges *et al.* 2019), that could affect negatively the growth of beneficial PCB-degrading bacteria. Indeed, when supplemented at increasing concentrations (0.25 mM-2 mM) to the bacterial strains *Acinetobacter calcoaceticus* P320 and *P. alcaliphila* JAB1, scopoletin inhibited cell growth while enhancing the ability of both the strains to form a biofilm, possibly activating this quorum sensing-mediated lifestyle to allow survival under stress. The identified exudates that increased their abundance in response to PCB-18 stress showed to sustain bacterial growth and enhance traits related to rhizocompetence: JAB1 and LB400 used hypoxanthine as unique carbon source and hypoxanthine also increased biofilm formation in JAB1. L-seryl-L-phenylalanine and L-arginil-L-valine were both utilized by LB400 as sole nitrogen sources.

5.2 Flavonoids improve traits involved in *P. xenovorans* LB400 rhizocompetence and early root colonization

Selected plant flavonoids were investigated to assess their influence on some rhizocompetence traits of the PCB-degrading bacterium *P. xenovorans* LB400, which are essential features to establish a stable association with the plant (Allard-Massicotte *et al.* 2016). As reported in the heat-map in Figure 1a, the assayed flavonoids selectively modulate the growth of strain LB400: in presence of naringin, the bacterial cells proliferated faster at

all assayed concentrations, with the higher maximum growth rate (+13.4%) at 50 µM compared to the control. Flavonoid-mediated improvement in the growth parameters corresponded to an increased bacterial biomass reached at the stationary phase after 24/30 hours of growth (Figure 1b). The higher yields were recorded for 20 µM flavanone and flavone (+9.2 and 15.3%, respectively), for 50 µM naringin and naringenin (+27.8 and 17.3%, respectively) and for 50 µM quercetin (+ 40.1%) compared to control.

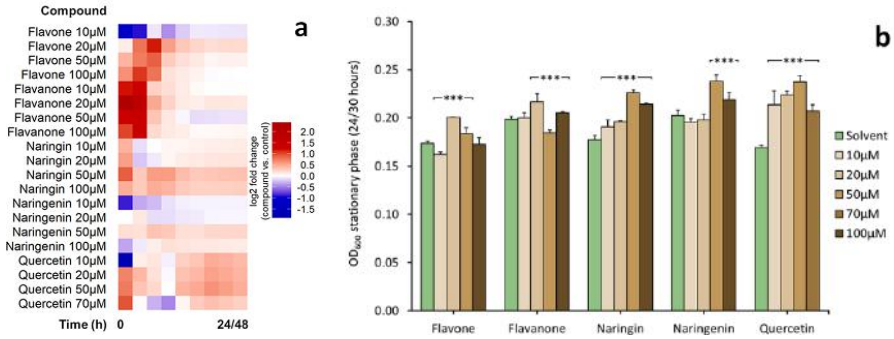

Figure 1 **(a)** *Relative growth of LB400 during 24/48 hours in presence of selected concentrations of flavonoids compared to the negative controls; red colour indicates positive increment of the log2 fold change while blue colour indicates decrement.* **(b)** *Bacterial biomass reached at stationary phase is expressed as OD_{600}. Bars represent the average ± standard deviation of 3 independent experiments. Statistical analysis was performed using Mann-Whitney test (***: $p \leq 0.001$).*

The flavonoids involved in growth stimulation of the strain also influenced functional traits, potentially stimulating the early recruitment of the bacterial cells by the plant and their adhesion to the root. Naringin at 50 µM was shown to influence bacterial swimming motility *in vitro* by increasing the swimming motility halo diameter by 6.6% compared to control, while flavone and quercetin inhibited the strain motility at the same concentrations. Interestingly, 50 mM quercetin and 50-100 mM naringenin had instead a role as chemo-attractants for LB400 revealed through gradient plate chemotaxis assay (Figure 2a). As reported in Figure 2b, biofilm formation ability, necessary for a stable colonization of the root over time (Knights *et al.* 2021), significantly increased 24h from inoculation in presence of 100 µM naringin and naringenin (+47% and +44% CV/OD_{600}, respectively).

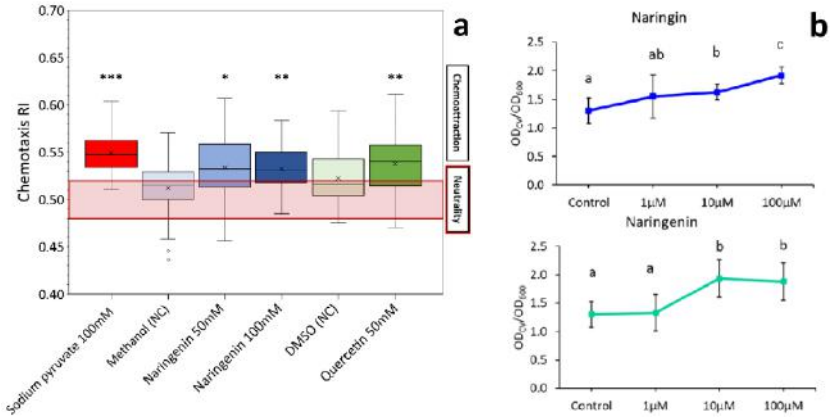

Figure 2 **(a)** *LB400 chemotaxis response index (RI) in presence of flavonoids, of negative controls (methanol and DMSO) and of 100 mM sodium pyruvate as positive control. The graph reports data from at least 3 independent experiments. Statistical analysis was performed using Tukey-Kramer's post-hoc test (*: $p \leq 0.05$, **: $p \leq 0.01$).* **(b)** *Biofilm formation of LB400 expressed as ratio between the crystal violet OD and the OD_{600} as index of bacterial growth. The graph reports data from 3 independent experiments. Statistical analysis was performed using Dunn's post-hoc test and letters indicate statistically different groups ($p \leq 0.05$).*

Considered flavonoids' influence in enhancing rhizocompetence traits of LB400, an early colonization assay was set up by dipping 6 days-old *Arabidopsis* plantlets for 1 hour in a bacterial solution containing 10^7 cells/mL. The results showed that the *Arabidopsis* mutant *tt8*, that over-accumulates flavonoid aglycones, including quercetin and naringin (Narasimhan *et al.* 2003), was significantly more colonized ($1.09x10^4$ cells/mg plant) than the *null* flavonoid mutant *tt4* ($5.42x10^3$ cells/mg plant), while in the WT the colonization efficiency recorded was $5.57x10^3$ cells/mg plant, potentially demonstrating a positive role of flavonoids in inducing bacterial early plant

colonization.

5.3 *Pseudomonas* JAB1 biosensor preparation and validation

The PCB-degrading strain *Pseudomonas* JAB1 was engineered with a plasmid containing a green fluorescent protein (eGFP) gene regulated by the promoter of the *bph* operon, which is involved in PCB degradation, obtaining a strain expressing fluorescence as a proxy for the activation of the PCB biodegradation pathway. The biosensor strain was validated by inducing the expression of the eGFP fluorescent protein with different concentrations of biphenyl (1 µM to 250 µM). By relating the relative fluorescence units (RFUs) measured through a spectrophotometer, to the bacterial growth (OD_{600}), it was observed that 2 hours after the induction the biosensor showed a significantly different emission of fluorescence compared to the non-induced control. This data suggested that the biosensor sensitivity is achieved with 10 µM biphenyl. Furthermore, the induction ratio of JAB1 biosensor (calculated as the ratio between RFU/OD_{600} of the induced cells and of the prior-to-induction cells) was seen to correlate linearly to the biphenyl concentrations tested. The induction of the expression of *bphA* by biphenyl was also verified via RT-qPCR, confirming a peak of relative expression at 2 hours after the treatment with biphenyl.

5.4 Analysis of the colonization pattern of *Arabidopsis* roots with fluorescent PCB-degrading bacteria

5.4.1. P. alcaliphila JAB1 supports the 'cry-for-help' hypothesis and *preferentially colonizes Arabidopsis root tip.* The strain JAB1 was tested for growth on *Arabidopsis* Col-0 root exudates and was observed capable of utilizing compounds present in root exudates as growth substrates. Moreover, the results of this experiment contributed to support the 'cry-for-help' hypothesis since the root exudates of plants exposed for 7 days to PCB stress promoted the growth of JAB1 more than the exudates released in control conditions, suggesting that the plant modulates its root exudation to counteract the stress and recruit beneficial bacteria. To understand the involvement of flavonoids, reported as key exudates for inducing the bacterial degradation of PCBs, on JAB1 biosensor root colonization ability, WT *Arabidopsis* (ecotype L*er*) and the flavonoid iper-producing mutant *tt8* were used. By observing the colonization pattern using fluorescence microscopy, the roots exposed to 20 µM PCB during *in vitro* plant growth resulted more intensely colonized than the non-stressed controls. Furthermore, the strain colonized more efficiently the *tt8* mutant, if compared with WT *Arabidopsis*, implying an involvement of flavonoids in recruiting the bacterium. The eGFP signal, indicating the induction of the biphenyl degradation pathway, was particularly visible in the root tip of PCB-stressed plants, as further confirmed by re-isolation of the strain from specific root sections. Confocal microscopy showed that JAB1 was peculiarly localized on the root cap, an unusual localization for plant-associated bacteria (Gamalero *et al.* 2005), indicating that the bacterium is adapted to this ecological niche. Moreover, the intense eGFP signal observed indicates that the root cap releases specific compounds that could induce the biphenyl catabolic pathway and potentially play a role in bacterial-driven PCB removal. *5.4.2. P. xenovorans LB400 promotes Arabidopsis but its long-term root colonization pattern is not influenced by flavonoid exudation*. The interaction between the PCB-degrading bacterium *P. xenovorans* LB400 and WT *Arabidopsis* (ecotype L*er*) and *Arabidopsis* mutants with an altered flavonoid exudation pattern was assayed *in vitro* in presence of 20 µM PCB-18 stress or in control conditions (acetone). The mutants included *tt4*, a *null* flavonoid producer, and two flavonoid overproducing lines, *tt8* (accumulating flavonoids aglycones in the roots) and *ttg1* (accumulating flavonoids and their conjugates). The results concerning plant fresh weight at day 14 of growth onto PCB-spiked medium showed that the stress caused by the contaminant had a major effect on the roots by significantly decreasing their fresh weight for all *Arabidopsis* lines (Figure 3a represents only the WT values, as an example). The shoot instead was not dramatically affected, especially in WT and *tt4* plants (Figure 3b). The presence of the bacterium induced growth promotion similarly for all the mutants compared to the mock control (no inoculation of LB400): plant root and shoot fresh weight was significantly higher in plants colonized by LB400 and generally corresponded also to an enhanced root length at day 7, both in presence and absence of PCB stress. Overall, these results suggest that LB400 exerts a growth-promoting activity when associated to *Arabidopsis*, but this does not depend on flavonoid exudation. When analyzing the colonization rate of LB400 on *Arabidopsis* lines at day 7 and at day 14 by re-isolation, no significant differences were visible between the mutant lines. Fluorescence microscopy observations using the *mScarlet*-tagged LB400 strain highlighted the ability of the bacterium to colonize diverse root zones in the WT and flavonoid mutants. These results are consistent with the *in vitro* tests analyzing the effect of flavonoids on rhizocompetence traits: flavonoids might in fact have an exclusive role in early colonization phases by having a priming effect, influencing bacterial motility and inducing chemotaxis. The long-term colonization might be uniform between all *Arabidopsis* lines because, once established on the rhizoplane, LB400 persistence on the roots could be tuned to other metabolites than flavonoids exuded by *Arabidopsis* mutants, that could reshape and influence LB400 abundance. For instance, *tt4* mutants do not release flavonoids but over-accumulate other secondary metabolites such as organic acids that could play a role in LB400 sustainment (Zhalnina *et al.* 2018). By testing the growth of LB400 on exudates collected from PCB-stressed or untreated *Arabidopsis* roots, results showed indeed that *tt4* exudates were also effective at supporting the growth of the bacterium (Figure 3c).

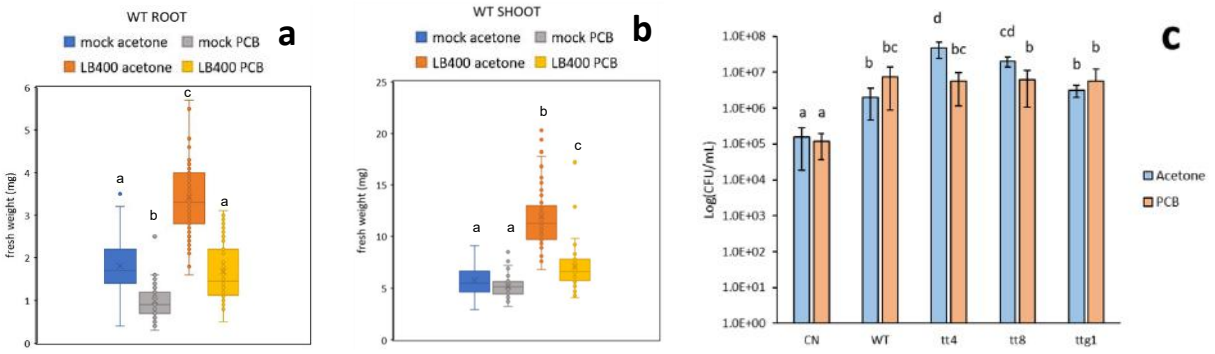

Figure 3 *Total root* **(a)** *and shoot* **(b)** *fresh weight of WT Arabidopsis in presence of PCB or in the untreated control (acetone) and in presence or absence (mock) of LB400. The graph reports data from 3 independent experiments.* **(c)** *LB400 growth on root exudates collected from WT Arabidopsis and flavonoid metabolic mutant lines at day 7 of treatment with PCB or acetone (untreated control). LB400 was inoculated at 5x10^{4}cells/mL and grown for 3 days. CN indicates ½ MS medium containing only acetone or 20 µM PCB-18. The bars represent the average ± standard deviation of 3 independent experiments. For all figures: letters indicate statistically different groups (Dunn's post-hoc test with p ≤ 0.05).*

6. Conclusions and Future Perspectives

The mechanisms that underlie the root-exudate mediated interactions between the host plant and the associated microbiota in the holobiont are not yet well understood. This knowledge could be useful in the perspective of applying bacteria that can promote plant growth or contribute effectively in boosting phyto-rhizoremediation strategies. In this work the role of flavonoids, key secondary metabolites released by the plant and well known to be involved in plant-rhizobia early interaction, was elucidated. Flavonoids can act as regulators of rhizocompetence-related traits during early root colonization also for non-rhizobia beneficial microorganisms, as the PCB-degrader *P. xenovorans* LB400. In addition, the results obtained allowed for a deeper understanding of the interactions between plants and beneficial bacteria, especially under stress conditions caused by PCBs, and to observe a case of putative 'cry-for-help' mechanism given by the presence of a soil contaminant, a topic not yet widely explored in literature. This knowledge could enable more targeted approaches in PCBs rhizoremediation, often affected by a limited efficiency, by providing useful insights for the possible exploitation of natural metabolites released by plant roots. Future research should be addressed at characterizing plants with specific root exudation patterns that could be used for *in vivo* rhizoremediation studies together with specific PCB-degrading bacteria to evaluate their efficacy in PCB clean-up.

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Metabolic attenuation of probiotics: a strategy for functional beverages development

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This PhD thesis dealt with the development of a strategy to counteract the deviation of probiotic food characteristics induced by probiotic metabolism. The strategy applied, namely metabolic attenuation, was based on a multiple physical approach, sonication and microencapsulation, to control the fermentative metabolism of the probiotic *Lacticaseibacillus casei* ATCC 393.

Attenuazione metabolica dei probiotici: una strategia per lo sviluppo di bevande funzionali

Questa tesi di dottorato ha riguardato lo sviluppo di una strategia per contrastare l'alterazione delle caratteristiche di un alimento probiotico indotte dal metabolismo del probiotico stesso. La strategia applicata, ovvero l'attenuazione metabolica, ha riguardato un approccio fisico multiplo, la sonicazione e la microincapsulazione, per controllare il metabolismo fermentativo del probiotico *Lacticaseibacillus casei* ATCC 393.

Key words: sonication, microencapsulation, flow cytometry, cultivability, acidification, surface properties.

1. Introduction

In accordance with the PhD thesis project previously described, this oral communication reports the main results of the following three activities:

A1) sonication process design and optimization and characterization of its effects on *L. casei* ATCC 393;
A3) microencapsulation process design and optimization;
A4) sonication and microencapsulation assessment as multiple strategy.

2. Sonication

Ultrasound has gained much attention in the last decade as a mean to manipulate microbial cells. Mechanical and chemical events (temperature and pressure increase, high shear forces and free radical generation) occur upon the implosion of the cavitation bubbles. Thus, the physiology of the cell is altered. The microorganism response, stimulation, inactivation, or destruction, depends on the intensity of the phenomenon of cavitation (Zupanc *et al*., 2019). So far, very little is known about the modulation of probiotic's activity and of the attenuation effect.

3. Microencapsulation

Microencapsulation is a well-known entrapment process used in the food and probiotic field. Microcapsule controls the rate of nutrient uptake and metabolite release, slowing them down enabling minimal cell-environment interactions (Sun *et al.*, 2023). From this point of view, microencapsulation can be considered an attenuation technology. To our knowledge, microencapsulation has never been used to modulate probiotic metabolism.

4. Experimental Procedure

In this PhD thesis the experimental procedure was set up by performing sonication experiments on the probiotic *L. casei* ATCC 393 followed the determination of its effects on several cell characteristics by applying conventional and multiparametric analysis. An independent microencapsulation experiment was then performed to choose the encapsulating agents and their concentrations. Finally, the two technologies were combined.

5. Materials and Methods

Two pulsed sonication treatments (6 or 8 min) with fixed power and frequency were carried out on water bacteria suspension. Attenuation was assessed as pH decrease of MRS broth after 6 and 24 h of incubation at 37 °C (Racioppo et al., 2017). Then, cultivability (spread plate count and growth index), auto-aggregation, hydrophobicity (cells affinity to iso-octane), membrane permeability and biofilm production were evaluated. Probiotic resuscitation through a growth curve was assessed. Light microscopy and light scattering angles were

used for a morphological characterization. SYTO24™ and cFDA (carboxyfluorescein diacetate) were combined with PI (propidium iodide) for viability and esterase activity evaluations through a flow cytometer. Alginate concentration (0.8, 1.0, 1.2, 1.5%) and chitosan coating (0.7%) were evaluated in the attenuation efficacy of microencapsulation. Microcapsules shape and size were also studied. Finally, sonicated cells were entrapped in 1.5% alginate and in chitosan-alginate microcapsules.

6. Results and Discussion

6.1 Sonication

6.1.1 Acidifying capabilities and cultivability

Table 1 shows the results of ultrasound-induced attenuation and probiotic cultivability. The 6 min-treatment induced temporary attenuation while the 8 min-treatment induced complete attenuation. The findings imply that LC_S6 can restore its metabolism and that increasing the sonication intensity results in a stronger attenuation effect. Hypothetically, free radicals, formed due to implosion of cavitation bubbles, can interact with enzymes involved in the sugar transport and metabolization system leading to their oxidation and, consequently, alteration of normal function.

Table 1 *Attenuation and cultivability alteration induced by ultrasound. LC_S0:* L. casei ATCC 393 *non sonicated (control); LC_S6:* L. casei ATCC 393 *sonicated for 6 min; LC_S8:* L. casei ATCC 393 *sonicated for 8 min.**

Sample	Acidification (ΔpH)		Log CFU/ml	Growth index		
	t_6	t_{24}		GI > 75 % No growth inhibition	25 < GI < 75 % Partial growth inhibition	GI < 25 % Complete growth inhibition
LC_S0	0.38 ± 0.05^{A}	2.16 ± 0.18^{A}	9.30 ± 0.02^{A}	-	-	-
LC_S6	0.06 ± 0.02^{B}	1.91 ± 0.12^{A}	8.58 ± 0.12^{B}	+	-	-
LC_S8	0.03 ± 0.02^{B}	0.97 ± 0.14^{B}	6.43 ± 0.04^{C}	-	-	+

Sonication reduced the plate count by approximately 1- and 3-Log for the 6 and 8 min-treatment, respectively. The growth index was calculated as follows:

$$Growth\ index\ (GI) = \frac{A_{US}}{A_C} \times 100 \quad (1)$$

where A_{US} is the absorbance of sonicated samples, and A_C is the absorbance of the control.

Collected data show that there was no growth inhibition for sample LC_S6 and complete inhibition in the case of LC_S8. Therefore, probiotic cultivability was affected by sonication. In stress conditions, bacteria can enter in a viable but non-culturable (VBNC) state. However, further analysis is required to proper define the ultrasound-induced VBNC status. Results obtained from the growth index analysis suggest that LC_S6 resuscitate. Although the more intense treatment may have caused LC_S8 inactivation, Brandão et al. (2021) demonstrated that ultrasound inactivation does not impair the health benefits of probiotics.

6.1.2 Cell surface characterization

Cell surface properties of *L. casei* ATCC 393 and of the sonicated probiotic are summarized in Table 2.

Membrane damage is given by:

$$Absorbance\ increase\ (AI)\ \% = \left[\frac{(A_{US} - A_C)}{A_C}\right] \times 100 \quad (2)$$

Auto-aggregation and hydrophobicity were calculated as follows:

$$AA\ or\ HY\ \% = \left[\frac{(A_0 - A_t)}{A_0}\right] \times 100 \quad (3)$$

where A_0 is the absorbance of samples at time 0, and A_t is the absorbance after incubation.

Biofilm production was quantified by establishing a low cut-off (ODc) and comparing OD_{570} of the samples with it. Sonicated *L. casei* ATCC 393 resulted in increased membrane permeability due its damage and weakening. Upon sonication, a negative correlation was found between sonication treatment and probiotic auto-aggregation, while hydrophobicity increased, and biofilm production improved. Our results suggest that ultrasound alters the surface structure of *L. casei* ATCC 393, thus affecting its normal function and physiological activities. This could explain the different adhesive properties of the sonicated strains. Furthermore, comparing our data with those in the literature, it is evident that the response to ultrasound treatment is strain- and species-specific.

Table 2 *Adhesive properties and membrane structure evaluation before (LC_S0) and after sonication (LC_S6: 6-min treatment; LC_S8: 8-min treatment).**

Sample	Auto-aggregation (%)	Hydrophobicity (%)	Biofilm production	Membrane permeability (%)	
				A_{260}	A_{280}
LC_S0	23.98 ± 1.32^{A}	6.29 ± 0.68^{A}	Weak (OD ≤ ODc)	-	-
LC_S6	3.39 ± 0.78^{B}	11.68 ± 2.65^{B}	Strong (4ODc < OD)	216 ± 8.16^{A}	140 ± 10.00^{A}
LC_S8	0.65 ± 0.26^{C}	15.01 ± 1.59^{B}	Strong (4ODc < OD)	256 ± 10.69^{B}	165 ± 9.64^{B}

6.1.3 Cell morphology

Microscope images (Figure 1) show the ultrasound-induced morphology variation. Sonicated bacteria presented a single cell morphology, resulted in a smaller rod cell compared to the *Streptobacillus* morphology of the control. FCM analysis also confirmed these changes. Forward Scatter (FSC) is related to the cell size and surface area while the Side Scatter (SSC) is related to the granularity or internal complexity. Both parameters were reduced in sonicated samples (Table 3) proving structure, thus changing the cell morphology, reducing internal complexity.

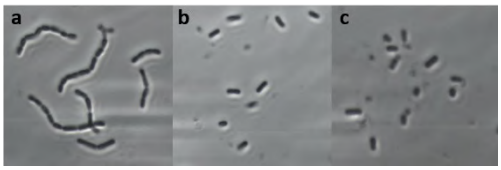


Figure 1 *Microscope images (400x magnification) of* Lacticaseibacillus casei *ATCC 393 (a);* L. casei *sonicated for 6 (b) and 8 min (c).*

Table 3 *Values and percentage decrease of* Lacticaseibacillus casei *ATCC 393 Forward and Side Scatter angles. LC_S0: L. casei non-sonicated (control); LC_S6: L. casei sonicated for 6 min; LC_S8: L. casei sonicated for 8 min.**

Sample	FSC	ΔFSC-H %	SSC	ΔSSC-H %
LC_S0	48,202 ± 268^{A}	-	30,817 ± 972^{A}	-
LC_S6	17,373 ± 177^{B}	-63.96 ± 0.57^{A}	10,247 ± 146^{B}	-66.74 ± 0.57^{A}
LC_S8	17,443 ± 443^{B}	-63.81 ± 1.12^{A}	9,840 ± 404^{B}	-68.07 ± 0.31^{A}

6.1.4 Membrane integrity and esterase activity

Stressful treatments generate subpopulations in different physiological states that can be detected by multiparametric flow cytometry analysis. As shown in Figure 2, the double staining with the cell permeant and impermeant nucleic acid dyes, SYTO 24$^{(TIM)}$ and PI, revealed a depletion of the viable population in both sonicated treatments in favour of dead subpopulation. In addition, sublethal injured cells were not detected. Our results suggested that the membrane of *L. casei* is impaired due to the violent events generated during ultrasound propagation and that ultrasound induced an "all-or-nothing" phenomenon. Figure 3 shows the distribution of metabolic active subpopulations in the suspension. The percentage of the cells able to metabolize the non-fluorescent cFDA in the fluorescent cF was reduced upon sonication. Thus, ultrasound affects the esterase activity of the probiotic.

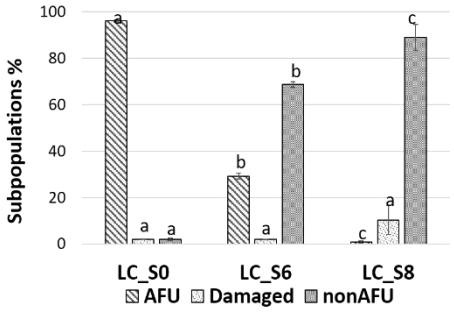


Figure 2 *Subpopulations detected with SYTO 24^{TIM}/PI double staining of* L. casei *before sonication (LC_S0) and after sonication (LC_S6: 6-min treatment; LC_S8: 8-min treatment). AFU (Active Fluorescent Unit): SYTO+/PI-; Damaged: SYTO+/PI+; nonAFU (non-Active Fluorescent Unit): SYTO-/PI+.*

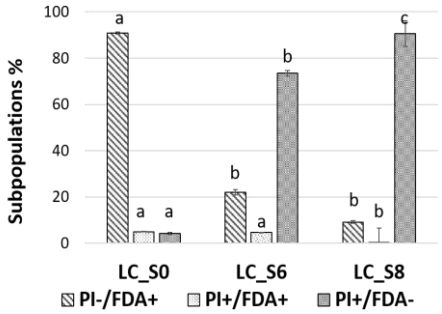


Figure 3 *Subpopulations detected with cFDA/PI double staining of* L. casei *before sonication (LC_S0) and after sonication (LC_S6: 6-min treatment; LC_S8: 8-min treatment). PI-/FDA+: metabolic active cells; PI+/FDA+: damaged cells; PI+/FDA-: non metabolic active cells.*

6.1.5 Comparison between cultivable, viable and metabolic active population

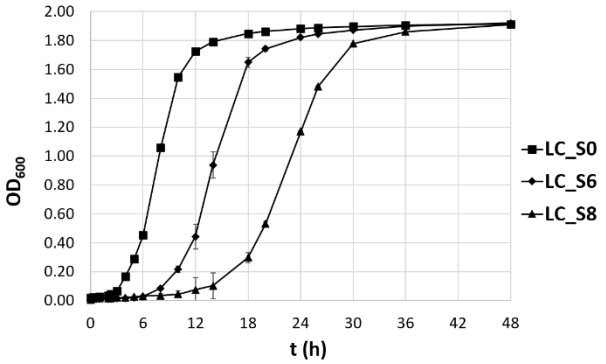
Multiple comparisons between the samples and between subpopulations of the same sample allows the discrimination of the ultrasound target on *L. casei*. Probiotic cultivability (Log CFU/ml), viability (Log AFU/ml) and metabolic activity (Log (PI-/FDA+)/ml) are summarized in Table 4. The 6-min treatment impaired the probiotic cultivability and metabolic activity, but not its viability. In addition, the three populations are not different. The higher intensity, instead, reduced the viability and cultivability while did not further impair the esterase activity. Furthermore, in LC_S8 the viable population is no different from the cultivable and metabolically active ones, while the cultivable population is lower than the metabolically active one. Therefore, *L. casei* retains its esterase activity upon each treatment, whereas it does not retain cultivability and viability with the severe one. Moreover, The FCM data negate the previous hypothesis of VBNC induction.

Table 4 *Comparison of viable, cultivable and metabolically active (Log) populations of the non-sonicated (LC_S0) and sonicated samples for 6 (LC_S6) and 8 min (LC_S8).**

Sample	Cultivability	Viability	Metabolic activity
LC_S0	9.15 ± 0.03^{Ab}	8.89 ± 0.03^{Aa}	8.86 ± 0.07^{Aab}
LC_S6	8.52 ± 0.08^{Ba}	8.60 ± 0.12^{Aa}	8.57 ± 0.06^{Ba}
LC_S8	6.37 ± 0.05^{Cab}	7.20 ± 0.23^{Ba}	8.36 ± 0.02^{Bac}

6.1.6 Restoring of normal physiology

Beyond the instantaneous effect of ultrasound on cultivability and viability, it could also affect the growth kinetics. The growth curve of the three samples is reported in Figure 4. Growth curves comparison revealed again a time-dependent effect of ultrasound on *L. casei* ATCC 393. A significant delay in the probiotic growth was found upon sonication. As postulated by Ojha et al., (2017), random and a high level of sonoporation lead to an uncontrolled efflux of cells components and to a delay of cell metabolism. These results also confirmed the growth index values.



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6.2 Microencapsulation

6.2.1 Microcapsules shape and size

The bead shape affects the mechanical and chemical stability of the microcapsules while the bead size affects the efflux rate of nutrients and metabolites. Taking this into account, these parameters were evaluated. As shown in Figure 5, by increasing the alginate concentration an improvement of beads shape is obtained, reaching a perfectly spherical shape and smooth surface with the 1.5% solution (MC). On the other hand, chitosan-alginate microcapsules (CMC) appear with a wrinkled and rougher surface (Figure 6). Moreover, MC are characterized

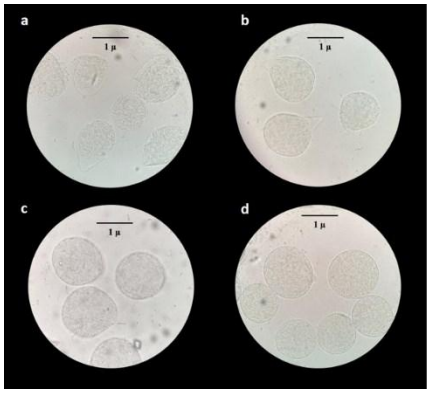

Figure 5 *Light microscope images (400x) of microcapsules with different alginate concentration. a: 0.8%; b: 1.0%; c: 1.2%; d: 1.5%.*

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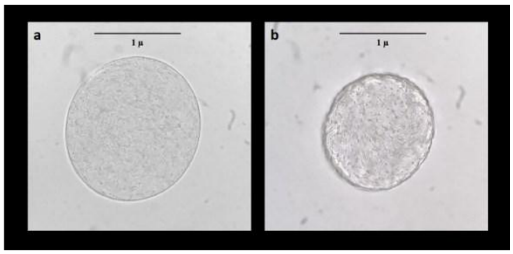

Figure 6 *Light microscope images (400x) of 1.5% alginate microcapsules (MC, a) and chitosan-alginate microcapsules (CMC, b).*

6.2.2 Attenuation efficacy of microencapsulation

Data of *L. casei* acidification abilities in microcapsules are reported in Table 5. Regardless the polymer concentration, the high-water content and high porosity of the alginate beads facilities the diffusion of molecules. Considering these results and the morphological evaluations, a chitosan coating was added on the 1.5% alginate microcapsules. Only, CMC were able to control the broth acidification. Alginate-chitosan high electrostatics interactions confer to the microcapsule new permeability properties, a more homogeneous surface and reduced porosity. Thus, the CMC capsule efficiently control the probiotic acidification abilities.

6.8 Sonication and microencapsulation as multiple attenuation strategy

Table 5 also shows the results of the efficacy of sonication and microencapsulation as an attenuation strategy. Collected data suggested that sonication-induced attenuation is significantly improved when a chitosan-alginate microcapsule is built around the cells. Interesting, chitosan-alginate microcapsules showed different preperformances from free cells and alginate microcapsules for all the samples.

Table 5 *pH decrease* of MRS broth after* Lacticaseibacillus casei *ATCC 393 inoculum in free form, in 1.5% alginate microcapsules (MC) and in chitosan-alginate microcapsules (CMC), not sonicated (LC_S0), 6-min sonicated (LC_S6), 8-min sonicated (LC_S8).*

Samples	Free form	MC	CMC
		6 h of incubation	
LC_S0	0.48 ± 0.03^{Aa}	0.26 ± 0.01^{Ab}	0.22 ± 0.05^{Ab}
LC_S6	0.14 ± 0.02^{Ba}	0.16 ± 0.01^{Ba}	0.16 ± 0.01^{Ba}
LC_S8	0.11 ± 0.03^{Ba}	0.15 ± 0.01^{Ba}	0.15 ± 0.01^{Ba}
		24 h of incubation	
LC_S0	2.17 ± 0.04^{Aa}	2.04 ± 0.05^{Aa}	1.54 ± 0.09^{Ab}
LC_S6	2.10 ± 0.02^{Aa}	1.83 ± 0.07^{Aa}	1.49 ± 0.04^{Ab}
LC_S8	1.04 ± 0.03^{Ba}	0.77 ± 0.01^{Bb}	0.31 ± 0.03^{Bc}

**Data are reported as means value ± standard deviation (n = 3). Statistical analysis (One-way ANOVA, unpaired and paired t-Student tests) were performed by SPSS software ($p < 0.05$). Different capital letters in the same column and different lower-case letters in the same row indicate that the differences are significant.*

7. Conclusion and Future Perspectives

The concept of healthy food for healthy life leads food companies and scientists towards the formulation of new probiotic foods that can meet the demand of the most (or all) consumers. Food probiotication need an in dept knowledge of the probiotic-matrix interaction. Research efforts have been focused on developing systems capable of preserving cell viability rather than preserving the sensory characteristics of the product. Therefore, the PhD thesis was focused on the development of an attenuation strategy to modulate or to control the metabolism of probiotic in food.

Our results showed that the attenuation effect of sonication depends on the intensity of the treatment. Ultrasound was proved to be a suitable technology to modulate the *L. casei* ATCC 393 acidification abilities. Although it caused some side effects such as a major loss of membrane integrity, reduction of cultivability and auto-aggregation, it also enhanced the surface hydrophobicity and biofilm production thus improving the probiotic adhesion abilities. This underline that ultrasound has a broad spectrum of action on bacteria. FCM data also proved that not all the metabolic activities of the cells are impaired. Therefore, although further analyses are needed, we can assume that not all cellular functions, such as probiotic activities, are impaired. Although modulation of probiotic metabolism occurs at different levels in the cell, the loss of viability does not allow to test more intense sonication treatments. Microencapsulation overcomes the limits of sonication in probiotic activity modulation. The findings of the study highlight that microcapsules with adequate barrier properties are the key factor to develop an efficient attenuation system. Besides the physical modulation, microencapsulation could modulate the bacteria Quorum Sensing (QS) activity (Li et al., 2023).

The results obtained in laboratory need to be confirmed in the more complex environment of a food matrix. The operating parameters for both sonication and microencapsulation can be changed and used in several combination. This opens up the possibility to build unlimited systems with specific attenuation abilities and to introduce a new kind of probiotic food on the market. Moreover, sonication and microencapsulation applications on bacteria are not limited on the metabolism attenuation. A study on transcriptome and on QS could lead to a deeper comprehension of the phenomena that occur inside the cells upon sonication and inside the microcapsule, thus allowing a conscious manipulation of both technologies to achieve many different goals.

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Sustainable Solutions in Technology and Quality Control of Olive Oil

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This PhD thesis deals with the set-up and application of rapid, innovative, and sustainable instrumental analytical methods for supporting the sensory analysis (Panel test) of virgin olive oils. Furthermore, it is focused on olive pomace valorization by developing sustainable methods for the extraction of phenolic compounds, followed by the characterization and shelf-life evaluation of the obtained extracts.

Soluzioni sostenibili nella tecnologia e nel controllo qualità dell'olio di oliva

Questa tesi di dottorato riguarda la messa a punto e l'applicazione di metodi analitici rapidi, innovativi e sostenibili, per la determinazione della qualità e purezza degli oli di oliva vergini come supporto all'analisi sensoriale. Inoltre, l'attività di ricerca è focalizzata sulla valorizzazione della sansa di oliva attraverso lo sviluppo di metodi sostenibili per l'estrazione di composti fenolici e le successive fasi di caratterizzazione e valutazione della shelf-life degli estratti ottenuti.

Key words: virgin olive oils; rapid analytical methods; Panel test; quality control; sustainability; olive pomace.

1. Introduction

This oral communication reports the main results of the following two research activities carried out in this PhD project: this oral communication reports the main results of the following four activities directed to:

A1) development and application of easy-to-use, innovative, rapid, and sustainable analytical instrumental methods to support the sensory analysis of virgin olive oils. In particular, a focus is carried out on the study of the volatile fraction by gas-chromatographic analyses and the composition by spectroscopic techniques, as a relevant potential tools in the determination of the commercial category of virgin olive oils;

A2) olive pomace valorisation: set-up of sustainable methods for extraction of phenolic compounds from olive pomace, as well as characterization and shelf-life evaluation of the so obtained phenolic extracts. This activity is aimed to produce extracts potentially usable in different industrial sectors, such as pharmaceutical, food and cosmetic.

2. Sustainability Aspects and Related Gaps in the Quality Control and Technology of Olive Oil Sector

Nowadays, one of the main worldwide challenges is the achievement of the 17 sustainable development goals, known as SDGs in the framework of UN Agenda 2030. Among these, it is important to mention the most related to this PhD project, the SDG 12, namely "responsible consumption and production", and specifically the target 12.3 which focuses on the halve per capita food waste at the retail and consumer level and reduce food losses along the food production and supply chains; and the target 12.4, that focuses on the management of chemicals and waste to significantly reduce their release to air, water and soil in order to minimize their adverse impacts on human health and the environment.

In the Mediterranean basin, olive oil represents one of the main food products, since more than 90% of the global production comes from this area, concentrating mainly on European countries like Spain, Greece, and Italy (European Commission, 2023). In the European Union, virgin olive oils (VOOs) can be classified in three commercial categories depending on their quality degree: extra virgin (EV), virgin (V) and lampante (L) (EU Reg. 2022/2104). The different quality level of each commercial category corresponds to different values and, subsequently, to various prices.

It is important to consider that most of the official analytical methods to assess the quality and genuineness of VOOs consist of time-consuming and complex procedures, often with the use of toxic chemicals and solvents which are dangerous for human health and the environment or are expensive and difficult to be managed. For these reasons, there is a strong and growing demand for rapid, easy-to use and environmentally friendly analytical procedures to support the official ones. This includes procedures that do not require solvents at all, such as the determination of volatile compounds by gas-chromatographic techniques with headspace-solid phase microextraction (HS-SPME-GC), ion mobility spectrometry (HS-GC-IMS) or HS-Flash-GC (Quintanilla-Casas *et al.*, 2020). It is well known that volatile compounds have a crucial role to determinate VOOs quality, since

they are directly responsible for the olfactory notes, and methods for their determination could be used as support for sensory analysis in the classification of VOO based on the quality grade (Barbieri *et al*., 2020a; Quintanilla-Casas *et al*., 2020).
Furthermore, regarding rapid, innovative, and sustainable techniques for the assessment of VOOs quality and genuineness, current investigations are also focused on the adoption of optical techniques (Delfino *et al*., 2018). In particular, NIR, MIR, Raman and FT-IR spectroscopic methods are useful tools for the rapid determination of food composition and molecular structure, also in the case of olive oils.
In the context of a sustainable olive oil production, olive pomace is the main residue in the mechanical extraction of the olive oil from the olive fruits and it is basically composed of skin, pulp and stone pieces, water, and oil. The major problem related to olive pomace is that it also contains organic compounds with phytotoxic properties, that are dangerous for the environment (Nunes *et al*., 2021). Although it represents an important environmental issue, olive pomace is also characterized by the presence of high added value molecules, such as phenolic compounds (Dermeche *et al*., 2013), widely recognised for their beneficial properties (e.g. antioxidant activity). For this reason, this by-product is a potential source of phenolic compounds and their valorisation as functional ingredients in pharmaceutical, cosmetic and food industries (Nunes *et al*., 2016) represents a promising sustainable valorisation strategy, especially in a circular economy perspective.

3. Experimental Procedure

Regarding the first activity (A1), the research has started with the collection of commercial VOOs. The sensory analysis (Panel test) was carried out by four panels to robustly determine the commercial category of each sampled oil, among EV, V and L. Secondly, the same sample set was analysed by gas chromatography coupled with ion mobility spectrometry (HS-GC-IMS) and Flash-gas chromatography (Flash-GC) to investigate the volatile fraction, since it is strictly correlated with the sensory attributes, both fruity and defects. The data obtained from the two techniques were elaborated by applying chemometric approaches, such as PLS-DA models, to predict the commercial category.
Finally, a selection of the same samples set was analysed by spectroscopic techniques (NIR, FT-IR and Raman), during a 3-months visiting period at Queen's University Belfast (from November 2022 to February 2023) to investigate composition and molecular structure of VOOs. Regarding data elaboration, a focus on rancid defect is under investigation, mainly applying PLS-DA models, since it is directly related to the oxidation status of olive oil (Frankel *et al*., 1983), and consequently to its quality. Also, a "data fusion" between the results of the different analytical techniques will be considered to obtain more robust predictive models.
Regarding the second activity (A2), the sustainable valorisation of olive pomace by obtaining extracts rich in phenolic compounds was carried out in the framework of the PRIMA project SUSTAINOLIVE "Novel approaches to promote the SUSTAInability of OLIVE cultivation in the Mediterranean" (Grant Agreement no. 813904, 2019 – 2023).
The research has started from the set-up of the analytical procedure for the extraction of phenolic compounds and the characterization of the phenolic profile of olive pomace samples. Subsequently, the activities were aimed to develop a sustainable procedure through the application of a mechanical approach, using less toxic solvents than those usually adopted such as food grade ethanol, to obtain phenolic hydroalcoholic extracts. On these extracts, a shelf-life study, including both sensory and instrumental evaluations, was carried out to investigate their stability over time.

4. Materials and Methods

4.1 Rapid and sustainable instrumental analytical methods to support the sensory analysis

In this context, Flash-GC (FGC-E-nose Heracles II, AlphaMos, Toulouse, F) and HS-GC-IMS (Flavourspec®, G.A.S. Dortmund, Dortmund, D) techniques were performed on 120 VOOs, collected in order to have a relevant and balanced variety in the commercial categories. The samples were assessed by sensory analysis (Panel test) carried out by 4 Italian panels, in order to have a robust sensory classification thanks to the application of a decision tree developed within the H2020 OLEUM project (Barbieri *et al*., 2020b). To predict the commercial category, previously developed chemometric approaches based on a PLS-DA models, both for Flash-GC (Barbieri *et al*., 2020a) and HS-GC-IMS (Valli *et al*., 2020), were applied on a selection of these samples. In particular, the models combined to assign the commercial category and classify the samples with a certain probability were: EV *vs* noEV; L *vs* noL; EV *vs* V; L *vs* V.
In addition, regarding the HS-GC-IMS, the whole sample set was analysed also using improved analytical conditions with respect to the published ones (Valli *et al*., 2020), in which sample conditioning and other analytical parameters were modified to improve the resolution and sensitivity of the method.
One hundred out of 120 total samples were analyzed also by spectroscopic techniques, namely NIR, FT-IR and Raman, with the aim to support sensory analysis and with a specific focus on rancid defect. NIR spectra were acquired by FT-NIR diffuse reflectance module (Nicolet iS50, Thermo Scientific, Waltham, Massachusetts,

USA), equipped with Ge coated KBr beam splitter and InGaAs (Indium Gallium Arsenide) detector. FT-IR analysis was conducted using FT-IR module (Nicolet iS50, Thermo Scientific, Waltham, Massachusetts, USA), equipped with a DTGS detector and KBr beam splitter. Finally, FT-Raman measurements were performed using a Raman Microscope (DXR2 Raman Microscope, Thermo Scientific, Waltham, Massachusetts, USA) operated with an excitation laser light of 785 nm.

4.2 Olive pomace valorization

After the set-up of a sustainable method for the extraction of phenolic compounds without the use of toxic solvents, a mechanical approach (using a lab scale screw-press) was applied on the olive pomace by adding a mixture of water and food grade ethanol (80:20 % v/v) and two types of samples were obtained: one more liquid drained from the lower part of the mill (named *SI*) and one drier from the frontal part (named *SF*). On these samples, including olive pomace as it is (named *TQ*), a study focused on the phenolic fraction was carried out.
The more liquid samples drained from the lower part of the mill (*SI*) has been selected as more suitable to obtain stable hydroalcoholic phenolic extracts. Subsequently, the technological conditions to obtain this extract were developed: the procedure included filtration of the olive pomace, evaporation, and addition of food grade ethanol. On the selected extract, the phenolic compounds characterization and the assessment of its stability during a shelf-life study were performed, including both sensory and instrumental evaluations. The latter concerned the characterization of the phenolic fraction by UHPLC-MS/MS, the determination of the sum of simple phenolic molecules after acid hydrolysis (by UHPLC-DAD) and the determination of the total reducing molecules content through the Folin-Ciocalteu method. On the other side, a sensory descriptive analysis was carried out by 8 panelists trained for VOO assessment, through an olfactory evaluation, and excluding the perception of ethanol.
In particular, the shelf-life study was performed on a monthly basis and for two months (T0, T1 and T2) on the extract stored at room temperature and in dark conditions.

5. Results and Discussion

5.1 Rapid and sustainable instrumental analytical methods to support the sensory analysis

At now the data elaboration was completed on a set composed of 52 VOOs samples classified into the commercial category (EV, V and L), using previously developed prediction approaches based on PLS-DA models, both for Flash-GC (Barbieri *et al.*, 2020a) and HS-GC-IMS (Valli *et al.*, 2020) data. The commercial category was assigned with a certain probability combining the 4 models: EV *vs* noEV (Fig. 1); L *vs* noL; EV *vs* V; L *vs* V.
The results show comparable effectiveness between these two techniques, and they are satisfactory in terms of percentage of correctly classified samples for the different commercial categories (Table 1) with respect to that established by Panel test through the decision tree (Barbieri *et al*., 2020b), confirming the robustness and capabilities of such developed models.

Table 1 *Flash-GC and HS-GC-IMS outcomes, in terms of samples correctly classified compared to the sensory assessment, by the prediction models.*

	Flash-GC		***HS-GC-IMS***	
COMMERCIAL CATEGORY	**SAMPLES CORRECTLY CLASSIFIED**	**%**	**SAMPLES CORRECTLY CLASSIFIED**	**%**
EV	16/17	94.1	15/17	88.2
V	17/19	89.5	17/19	84.2
L	13/16	81.3	15/16	93.8
TOTAL	46/52	88.5	47/52	90.4

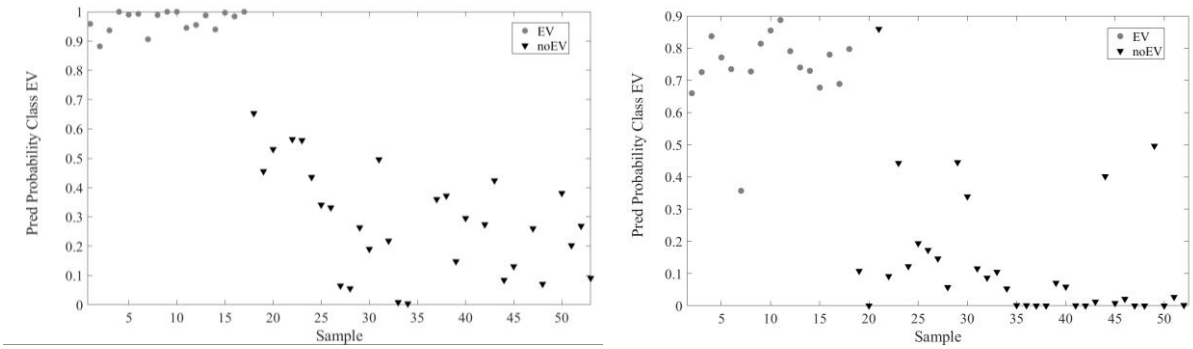

Figure 1 *Graphical results regarding the values of the class prediction probability by the single model EV vs. noEV, for both Flash-GC (on the left) and HS-GC-IMS (on the right). EV samples: grey circles; noEV samples: black triangles.*

The other 68 VOOs samples were also analyzed by sensory analysis (Panel test) and by both the abovementioned instrumental techniques, and the data elaboration is still ongoing.
In addition, regarding HS-GC-IMS technique, data elaboration on the results obtained using the improved analytical conditions with respect to the published ones (Valli *et al*., 2020) for the 120 VOOs is also at now ongoing.

5.2 Olive pomace valorization

The results show that the extract obtained from the olive pomace drained from the central part of the lab-scale mill, *SI*, is the richest in the concentration of both total reducing molecules, including the phenolic compounds, and detected simple phenolic molecules after hydrolysis (Table 2). For this reason, it has been selected as the most suitable sample as phenolic hydroalcoholic extract.

Table 2 *Average concentrations and relative standard deviations in the extracts obtained from the sample TQ, SI, SF (see the description of these samples in the paragraph 4.2). In the first column the total concentrations of the sum of unknown compounds (Unk), hydroxytyrosol (HTyr), and tyrosol (Tyr), obtained after acid hydrolysis of the extracts, are reported. In the third column, the concentrations in total reducing molecules contents by Folin-Ciocalteu method are reported. Statistical analysis: ANOVA, HSD Tukey, $p < 0.05$.*

Sample	Concentration (g Tyr + HTyr + Unk/kg olive pomace)	SD	Concentration (g gallic acid/kg olive pomace)	SD
TQ	1.12^{b}	0.25	3.29^{b}	0.38
SI	1.54^{a}	0.14	7.10^{a}	0.42
SF	0.64^{c}	0.02	3.09^{b}	0.39

Despite the statistic shows some significant differences among the three samples T0, T1, and T2, a clear reduction in the phenolic contents during the two months of the shelf-life is not observed (Table 3).

Table 3 *Average concentrations and relative standard deviations of the extracts from the sample T0, T1, T2 (see the description of these samples in the paragraph 4.2). In the first column the total concentrations of the sum of unknown compounds (Unk), hydroxytyrosol (HTyr), and tyrosol (Tyr), obtained after acid hydrolysis of the extracts, are reported. In the third column, the concentration in total reducing molecules contents by Folin-Ciocalteu method are reported. Statistical analysis: ANOVA, HSD Tukey, $p < 0.05$.*

Sample	Concentration (mg Tyr + HTyr + Unk/mL extract)	SD	Concentration (mg gallic acid/mL extract)	SD
T0	0.23^{b}	0.00	0.55^{c}	0.01
T1	0.25^{a}	0.00	0.63^{a}	0.02
T2	$0.24^{a,b}$	0.00	0.60^{b}	0.01

Regarding the UHPLC-MS/MS analytical approach, data elaboration is still ongoing to investigate the phenolic profile of the extracts.
Moreover, a sensory descriptive analysis was carried out by 8 panelists trained for olive oil assessment, through only an olfactory evaluation, and they were asked to exclude the perception of ethanol in their assessment. Defects or other negative attributes were not perceived. The attributes were all considered as positive and related mostly to specific notes resembling vanilla, caramel, red fruits, and olive fruits.

6. Conclusions and Future Perspectives

Most of the VOOs were correctly classified by the predictive models, according to the commercial category obtained by the application of a decision tree on the sensory results of four different panels. The two analytical approaches (HS-GC-IMS and Flash GC) both showed effectiveness as potential instrumental screening methods to support the Panel test: this is confirmed also by the comparison of the satisfactory percentages of samples herein correctly classified with those obtained in external validations from the previous studies conducted by Barbieri *et al.* (2020) and Valli *et al*. (2020). For this reason, the adoption of such rapid and innovative gas chromatographic techniques could represent a potential useful tool to support the sensory analysis for the determination of the commercial category of VOOs, thus pre-classifying some samples, and simplifying the quality control work of the laboratories and companies in the olive oil sector. In fact, the concrete applicability of such rapid screening methods to predict the commercial category of VOOs could reduce the number of samples to be assessed by Panel test. Although these encouraging results, further efforts to improve the robustness of the models, i.e. by increasing the dataset, are needed. Moreover, the elaboration of the obtained results by a new HS-GC-IMS method with improved analytical conditions is now ongoing. In addition, also rapid spectroscopic techniques (FT-IR, NIR and Raman) were applied to a selection of the same sample set to

investigate their ability to support the sensory analysis as well, and the results are now under investigation. Finally, further studies are considering innovative chemometric approaches, such as the "data fusion" between the results obtained with the different analytical techniques adopted during this PhD project.
The second activity, related to the olive pomace valorization, was aimed to obtain phenolic hydroalcoholic extracts. Researchers, food industries and stakeholders are paying more and more attention to waste and by-products especially when they represent a source of bioactive molecules, such as olive pomace. Within this PhD research work it has been developed a sustainable phenolic extraction procedure (i.e. by a mechanical approach), using less toxic solvents, such as food grade ethanol. The obtained extract, during the shelf-life study, demonstrates stability over time, since a relevant reduction of the phenolic content was not observed, nor sensory defects or other negative sensory attributes were perceived. Thus, this research could contribute to improve the valorization of olive oil pomace, well-known for its high content of phenolic compounds, known for their beneficial properties, such as the antioxidant activity; in fact, extracts potentially usable in different industrial sectors, such as pharmaceutical, food and cosmetic, were obtained and the phenolic fraction will be soon completely characterized.
In conclusion, the research activities in this PhD project are providing sustainable solutions in relation to technology and quality control of olive oils.

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Development of active antioxidant packaging to preserve the nutritional quality of minimally processed produce

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This PhD thesis dealt with the assessment of the poorly understood concept in the food packaging domain that "for the packaging to be effective in the shelf-life extension of the product, the release kinetics of the active compound from active packaging must be of the same magnitude as of decay kinetics of the food to be preserved". This project aimed to understand the release kinetics phenomenon and how bioactive release behavior can be modeled using a suitable mathematical model. Furthermore, the release behavior was correlated with decay kinetics of the minimally processed F&V using in-vitro and in-silico approaches.

Sviluppo di imballaggi antiossidanti attivi per preservare la qualità nutrizionale dei prodotti minimamente lavorati

Questa tesi si è occupata della valutazione del concetto poco compreso nel campo dell'imballaggio alimentare secondo cui "affinché l'imballaggio sia efficace nell'estensione della durata di conservazione del prodotto, la cinetica di rilascio del composto attivo dall'imballaggio attivo deve essere della stessa grandezza come cinetica di decadimento dell'alimento da conservare". Questo progetto mirava a comprendere il fenomeno della cinetica di rilascio e il modo in cui il comportamento di rilascio bioattivo può essere modellato utilizzando un modello matematico adeguato. Inoltre, il comportamento di rilascio è stato correlato con la cinetica di decadimento dell'F&V minimamente elaborato utilizzando approcci in-vitro e in-silico.
Keywords: Release kinetics; decay kinetics; antioxidant packaging; mathematical modeling; polyphenol oxidase; molecular docking.

1. Introduction

Minimally processed fruits and vegetables (F&V) are those food products that are altered physically from their original state but remain in a fresh form. Over the years, the demand for consumption of minimally processed F&V has increased due to change in consumer's requirements for convenient, healthy, and fresh foods. However, minimally processed produce is more perishable as compared to original raw materials and have a shelf life of several days as compared to several weeks or months of raw produce due to the presence of cut surfaces, active metabolism of tissues, microbial growth due to cross-contamination and removal of the outer protective layer. Considering the above facts, a holistic approach in terms of the development of an active packaging form is required to preserve the intrinsic quality of fresh produce. The main challenges for the implementation of the new technology to real food is related to the complexity of the food systems and to the highly product-specific packaging parameters (Khan et al., 2021). Furthermore, for the packaging to be effective in shelf-life extension of the product, the release kinetics of the active compound from active packaging must be of the same magnitude as of decay kinetics of the food to be preserved. Thus, an adequate knowledge of the release kinetics and how it can be used to slow down the decay by the active compound is essential to design an antioxidant package by using mathematical modelling. The aim of this PhD project is to design an active antioxidant package capable of preserving the nutritional quality of the food product. In this context the work was divided into four main activities:
A1) Investigating the release kinetics of active compounds from active packaging
A2) Modelling the release behavior using mathematical modeling
A3) Correlating the release kinetics with decay kinetics of minimally processed fruits and vegetables (F&V)
A4) Employing an in-silico approach to elucidate the mechanism of inhibition of oxidative enzyme (in case of reduced lower oxidative enzyme activity):

2. Materials and Methods

2.1 Investigating the release kinetics of active compounds from active packaging

Initially, a computational methodology was followed to understand the interaction mechanism between

caseinate and a group of hydroxybenzoic acids, and the bioactive compound (i.e., gallic acid) with the best binding affinity was selected for further evaluation for in-vitro release kinetics according to EC regulation 10/2011. Furthermore, guar gum was also added to the mixture and gallic acid was used at different concentrations in the film-forming solutions (FFS). Another study was carried out to evaluate the impact of the inclusion of fennel (FEN) and coffee humic (COF) substances on the structure, physical, and release properties of active substances from composite materials based on sodium caseinate, guar gum, and beeswax. Ferulic acid displays poor thermal resistance during extrusion and compression moulding, slow 2,2-diphenyl-1-picrylhydrazyl (DPPH) reaction kinetics, and undetected release from polylactide (PLA) and polyhydroxyalkanoates (PHA)-based films into polar media. Thus, in this study, a ferulic acid derivative Bis-O-dihydroferuloyl-1,4-butanediol (BDF) was used as an active additive (up to 40 w%) in PLA, poly(3-hydroxybutyrate) (PHB), and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) matrices to produce blends by extrusion. These blends were then used to prepare films by solvent casting.

2.2 Modelling the release behavior using mathematical modeling

The mathematical models are useful in describing the release behavior of a bioactive from the polymeric chains of a film into the food simulant by using Fick's Second Law. The migration process was elucidated by the diffusion coefficient (D) and partition coefficient (K) of the migrant molecules. The Fick's Model, boundary conditions, and differential equations are as follows:

$$\frac{\partial C(x,t)}{\partial t} = D\left[\frac{\partial^2 C(x,t)}{\partial x^2}\right] \quad (1)$$

$$\begin{cases} \left.\frac{dc_i(t)}{d_t}\right|_{i=0} = 0 \\ \left.\frac{dc_i(t)}{d_t}\right|_{i=n} = 0 \end{cases} \quad (2)$$

Whereas C is the ratio of the concentration of bioactive at time t and its concentration after infinity. Furthermore, to simplify the solution of PDE of the Fick's Law, the method of lines was used with respect to the spatial variable on the second derivative. This approximation transforms the PDE into an ordinary differential equation (ODE):

$$\frac{dC_i t}{dt} = D\frac{(C_{i-1}(t) - 2C_i(t) + C_{i+1}(t))}{x^2} \quad (3)$$

x is the distance from the interface obtained by dividing the thickness of the film (e) to the total number of layers (η).

$$C = \frac{M_{f,t}}{M_{f,\infty}} \quad (4)$$

$$x = \frac{e}{\eta} \quad (5)$$

For ensuring the validation of mathematical models and to ensure the goodness of fit of the predicted data with experimental by minimizing the sum of square of the differences between measured and predicted values, root mean square error (RMSE) was calculated by using MATLAB (version R2022a, MathWorks, USA).

2.3 Correlating the release kinetics with decay kinetics of minimally processed fruits and vegetables

The control (PHBV) and active packaging films (PHBV.BDF 10%, PHBV.BDF 20%) were prepared from extruded polymeric blends by the solvent casting method used previously by Khan et al. (2023). Different parameters were evaluated during storage of the product wrapped in the packaging films i.e., weight loss, color properties, ascorbic acid content, total polyphenol content by Folin's method, polyphenol oxidase content by using a wet chemistry method.

It is essential to understand the degradation kinetics of ascorbic acid to predict quality losses during storage and the effect of release kinetics on the decay kinetics phenomenon. Thus, vitamin C degradation was described by zero-order (equation 6) and first-order models (equation 7):

$P = P0 - kt$ (6)

$P = P0 \exp(-kt)$ (7)

Where P is the measured vitamin C content at time t, P0 is the initial ascorbic acid content, and k is the rate change constant. The quality of regression and fitted models was determined by the coefficient of correlation (R^2).

2.4 Employing an in-silico approach to elucidate the mechanism of inhibition of oxidative enzymes

Molecular docking methods were used to study initially the interactions between gallic acid and PPO/AO (Zhou et al., 2016). To explore the reason for PPO inhibition caused by active PHBV-releasing films, molecular modeling was used between PPO and BDF molecules to understand the detailed interactions, stability of the protein/ligand complex, and mechanisms of inhibition (Zhou et al., 2016).

3. Results and Discussion

3.1 Release kinetics and mathematical modelling

In case of caseinate films, during the first 6 h of incubation, the concentration of released gallic acid was 171.76 ± 18.21 µg/ml, with a threefold increase (~624 µg/ml) in concentration after 48 h (Fig. 1). However, after 120 h, a non-significant increase in concentration was observed indicating towards equilibrium stage, which can be better defined in terms of the "swelling-controlled" model. In this study, ~26% of the gallic acid leached out into the food simulant, which could be due to migrant polarity similar to that of the food simulant and swelling of the polymer in the presence of the simulant. On the other hand, the addition of guar gum and different gallic acid concentrations also effected the release behaviour of gallic acid from the films. For instance, the gallic acid released from the films GAI*60 µg.ml^{-1}, GAII*250 µg.ml^{-1} and GAIII*650 µg.ml^{-1} was 67%, 32%, and 30% respectively. Similarly, the diffusion coefficient was also affected by an increase in the concentration and was: 8.10×10^{-12} m^{2}s^{-1}, 6.23×10^{-12} m^{2}s^{-1}, and 4.5×10^{-12} m^{2}s^{-1} for GAI, GAII, and GAIII films respectively. Because of the hydrophilic nature of the packaging films another set of polymeric films were prepared with a novel ferulic acid derivative such as Bis-O-dihydroferuloyl-1,4-butanediol (BDF) for food applications. the films with low BDF concentration (i.e., 10–20 w%) displayed a higher release percentage ($p < 0.05$) as compared to films with the highest BDF content (40 w%). BDF is hydrophobic in nature thus a lower amount of BDF in the films means it has more affinity for hydrophilic food simulant (i.e., 10% ethanol), thus more release of BDF can be expected from films with lower BDF content. Furthermore, a higher percentage of BDF (40%) favoured the BDF-BDF interaction rather than the BDF-PHA interaction along with the formation of a crosslinking cluster of PHA-BDF-BDF-PHA structure that leaves the BDF trapped and then delays its release (Fig. 2).

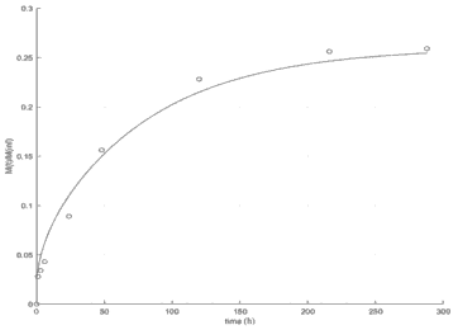

Figure 1 *Release kinetics of gallic acid from caseinate films*

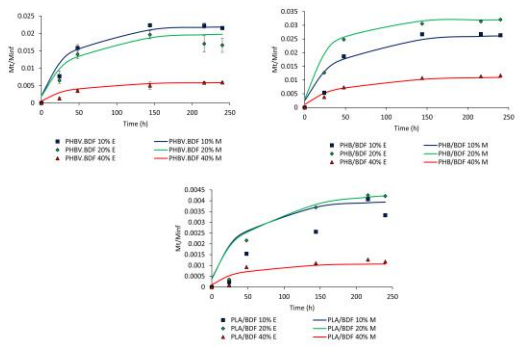

Figure 2 *Release kinetics of BDF from PHBV, PHB, and PLA films*

3.2 Combining release and decay kinetics

Based on the release data acquired previously and film integrity active PHBV films were selected for application on minimally processed apples. The vitamin C degraded in all the apple slices irrespective of the treatment used throughout the storage period. However, there was a significant difference ($p < 0.05$) in the ascorbic acid contents of apple slices packed in active packaging and the control samples (Fig. 3). Figure 4 shows the PPO activity as a function of treatment and time. Although all the apple slices packed in different

treatments displayed an increase in PPO activity throughout the storage period, however, the rate of increase of PPO activity was significantly faster ($p < 0.05$) in the un-packed apple slices or the ones packed in plain PHBV films mainly because PPO quickly utilized the substrate on apple surface. Ferulic acid derivative can either effectively decrease the reaction quinones produced (by donating electrons to PPO from their hydroxyl groups) during PPO-catalyzed oxidation of polyphenols or by cross-linking PPO through hydrogen bonding and π-π stacking interactions which can change the PPO polarity and reduce the brown-pigment formation. The lowest and highest TPC values (544.8±14.6 and 608.5±8.4 mgGAE/100g FW) were observed for apple slices unpacked and packed in 20% BDF-containing films (Fig. 5); these results can be directly correlated with the PPO activity since PPO enzymes are directly responsible for phenolic oxidation and degradation to produce brown pigments. PCA was used to uncover underlying mechanisms on how the different parameters i.e., weight loss, color parameters (a and L-value), PPO activity, and TPC are related to each other, and to determine how the treatments can be compared when taking into account these parameters simultaneously (Fig. 6).

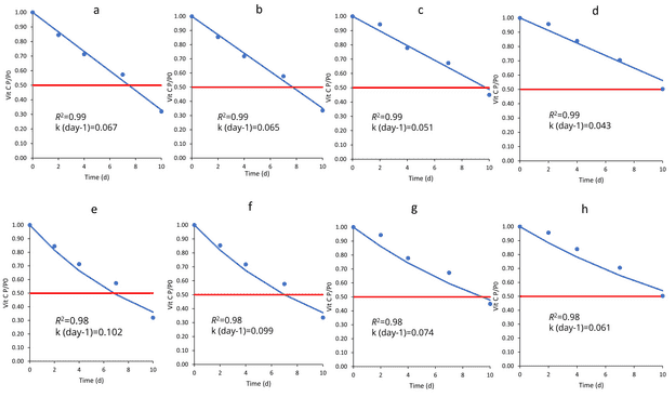

Figure 3 *Decay kinetics of ascorbic acid of apple slices wrapped in different packaging materials whereas a-d) zero order decay and e-h) first order decay modelling of ascorbic acid from samples recovered without film, plain PHBV film, PHBV.BDF 10%, PHBV.BDF 20% respectively (dots represent experimental and line represent model used for fitting)*

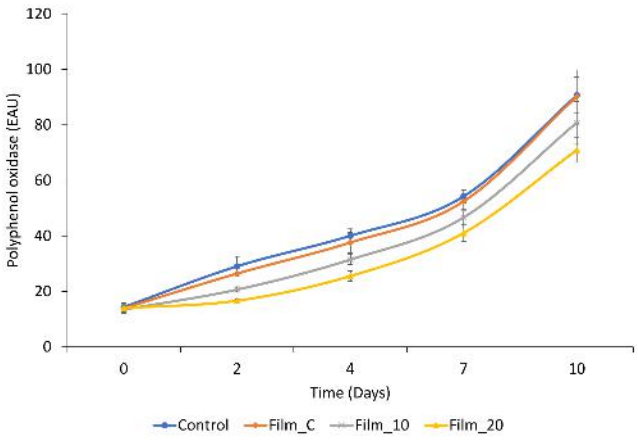

Figure 4 *PPO activity of the samples without film (control), plain film (Film_C), PHBV.BDF 10% (Film_10), and PHBV.BDF 20% (Film_20)*

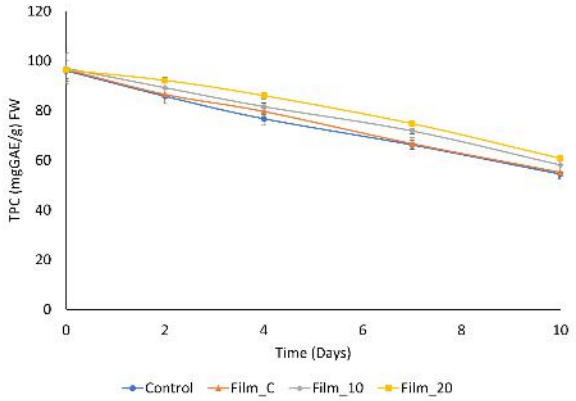

Figure 5 *TPC of the samples without film (control), plain film (Film_C), PHBV.BDF 10% (Film_10), and PHBV.BDF 20% (Film_20)*

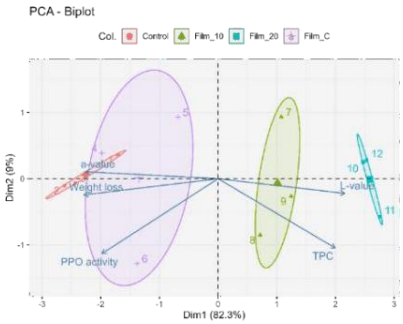

Figure 6 *PCA biplot of the nutritional quality parameters of apple slices packed in different films*

3.3 Inhibition mechanism exploration via in-silico study

Molecular modelling of BDF with PPO suggested a binding energy of -5.9 kcal/mol. Pi-Pi stacking was observed between benzene ring of BDF and PHE492 of the PPO enzyme which changed the polarity of the enzyme by causing rearranmnet of the secondary structure ultimately avoiding the formation of oxidation product as also observed previoulsy in in-vitro trials. Hence it was proved that BDF has the potential to inhibit the activity of PPO by binding near the active site (especially formation of hydrogen bonds could reduce the overall polarity of the enzyme).

4. Conclusions and Future Perspectives

While most of the literature available has focused on the effect of antioxidant packaging on lipid still there is limited information about the impact of antioxidant packaging on the quality of fresh and minimally processed F&V during shelf- life especially the loss of nutritional quality due to oxidative enzyme activity. Initially, casein-based systems were explored for release kinetics however their hydrophilic nature restricted their potential for packaging purposes. A comparative study between microbial origin-biopolymers and poly (lactide)s was carried out and PHBV-based systems displayed better release and structural integrity Ferulic acid derivative (BDF) effectively decreased the reaction quinones produced (by donating electrons to PPO from their hydroxyl groups) during PPO-catalyzed oxidation of polyphenols. This thesis provided evidence that release kinetics can be effectively used as an indicator for managing decay of the product. However, future studies are still required to improve the mechanical behavior of PHBV films; to explore the leeching phenomenon in detail, or the regulatory status of using ferulic acid derivatives in releasing packaging systems.

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Metabolomics to investigate the effects of treatments on food and of food consumption on health

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This PhD thesis aimed to advance the knowledge of the effects of treatment on food and food consumption on human health through metabolomics approaches. To this purpose, a specific SOP (Standard operation procedures) was set up and applied to deal with the metabolites of different food matrix. Then, metabolomics-oriented experiments focusing on the consequences of high hydrostatic pressure (HHP) on seafood products were carried out to investigate the effect of HHP on metabolic profile of seafood. Finally, a further metabolomics-oriented experiment targeted the potential mechanisms underlying the effect of a multistrain probiotic on cirrhotic patients.

La metabolomica per studiare gli effetti dei trattamenti sugli alimenti e del loro consumo sulla salute

Questa tesi di dottorato mirava a far progredire la conoscenza degli effetti del trattamento sugli alimenti e del loro consumo sulla salute umana attraverso approcci metabolomici. A tal fine, è stata messa a punto e applicata una specifica SOP (Standard operation procedures) per trattare i metaboliti di diverse matrici alimentari. In seguito, sono stati condotti esperimenti orientati alla metabolomica, incentrati sulle conseguenze dell'alta pressione idrostatica (HHP) sui prodotti ittici, per studiare l'effetto dell'HHP sul profilo metabolico dei frutti di mare. Infine, un ulteriore esperimento orientato alla metabolomica ha riguardato i potenziali meccanismi alla base dell'effetto di un probiotico multistrato sui pazienti cirrotici.

Key words: Metabolomics; High hydrostatic pressure; ^{1}H-NMR, probiotic, human health.

1. Introduction

Following the Ph.D. thesis project previously described (Lan, 2021), this oral communication reports the main results of the following four activities directed to:

A1) Literature review of the latest research related to investigating the effects of stress conditions on the physiological response and metabolism of food.
A2) Set up and application of specific NMR SOP to deal with the metabolites of seafood.
A3) Metabolomics-oriented experiments focus on the consequences of treatments on food composition and quality of treatments. The effects of HHP on the metabolism of grey mullet *(Mugil cephalus*), striped prawn *(Melicertus kerathurus*), and deep-water rose shrimp (*Parapenaeus longirostris*) during chilled storage were investigated.
A4) Metabolomics-oriented experiments focusing on the relationship between food composition and health.

2. Applications of metabolomics

Metabolomics, the field of research dedicated to studying the complete collection of small metabolites in a biological system (known as the metabolome), has broad applications. In the context of food, metabolomics provides a systematic approach to identifying and quantifying its components. This enables us to understand the chemical and biochemical changes that occur due to technological processes or microbial activity. These changes ultimately determine important product characteristics, such as nutritional quality, safety, and sensory attributes. By monitoring changes in the entire food matrix, metabolomic approaches can provide valuable insights into the impact of various food traits and transformations on consumer acceptability. Moreover, since the composition of metabolites in food directly affects human health upon consumption, a comprehensive examination of food intake through metabolomic analysis can include studying the metabolite profile of body fluids (Trimigno, et al., 2020). One of the preferred analytical platforms for investigating the metabolome of both food and human biofluids simultaneously is proton high-resolution nuclear magnetic resonance (^{1}H-NMR). Its effectiveness in metabolomics research is evident in studies such as that of Yang et al. (2020), where they employed 1H-NMR to quantify taste-active metabolites and explore taste variations in different Chinese sauce-stewed beef. Similarly, Trimigno et al. (2020) utilized ^{1}H-NMR spectroscopy to compare the metabolic effects of a nutritionally healthy New Nordic Diet with an Average Danish Diet by analyzing alterations in the human urine metabolome. Thus, ^{1}H-NMR has emerged as a valuable tool for capturing information about changes in food quality resulting from various treatments, as well as understanding the connections between food composition and human health.

Regarding food processing, non-thermal technologies are gaining popularity in the field of food processing, particularly in developed countries. These technologies offer milder treatment conditions compared to heat exchange, resulting in higher quality while ensuring food safety. Among these technologies, high hydrostatic pressure (HHP) has been proven effective for preserving various seafood products by inhibiting the growth of undesirable spoilage microorganisms (Ekonomou & Boziaris, 2021). Metabolomics plays a crucial role in this context as it provides valuable insights into the consequences of microbial growth on sensory characteristics such as freshness and flavors, by tracking changes in the metabolome of fish flesh (Lou et al., 2020). For instance, the concentrations of adenosine triphosphate (ATP) and its breakdown products, including adenosine-5-diphosphate (ADP), adenosine-5-monophosphate (AMP), inosine-5-monophosphate (IMP), inosine, and hypoxanthine, serve as indicators of freshness in various seafood. Additionally, certain water-soluble, low-weight molecules are recognized as taste-active compounds contributing to specific flavors in seafood, categorized as umami, sweet, sour, and bitter (Nishimura & Kato, 1988). However, since the effects of HHP on seafood can vary and depend on both process parameters and seafood species (Puértolas & Lavilla, 2020), further research is necessary to gain a deeper understanding of how HHP affects specific seafood metabolomes.
The application of metabolomics in the study of probiotics and their impact on the human body in the context of nutrition is an intriguing research area. Probiotic bacteria are utilized in the production of functional foods to promote a healthy diet, leveraging their positive effects on the immune system and overall well-being. Remarkably, the mechanisms through which probiotics exert their health benefits remain largely unexplored. This holds true for various applications, including one examined by Román et al. (2019), who conducted a double-blind, placebo-controlled, randomized clinical trial to investigate the effects of a multistrain probiotic on individuals with cirrhosis.

2. Materials and Methods

2.1 Effects of HHP treatment on the metabolic profile of seafood products

HHP treatment

Striped prawns, rose shrimps, and grey mullets were fished in the Adriatic Sea. They were fast frozen at a temperature of -18°C for 24 h by the company Economia del Mare (Cesenatico, Italy). Seafood samples were subjected to mechanical deboning and shell removal after thawing at 4°C for 16 h. Flesh was manually cut into pieces and packed in polypropylene (PP) trays containing 6 monoportions of about 15-20 g each that were packed under vacuum with a PP film. Vacuum packed samples were subjected to HHP treatments (400, 500, and 600 MPa) for 10 min performed by the company HPP Italia s.r.l (Parma, Italy). The untreated sample was used as a control.

Storage

After treatment, samples were stored at 2±1°C and microbial shelf life ended when reaching a microbial load of 6 log CFU/g. During storage, samples were subjected to analytical determinations after 1, 6, 9, 14, 21, 28, and 35 days. For each HHP treatment and at each storage time, 3 different packages were used.

Microorganisms analysis

All the samples were investigated for the presence of *Salmonella spp*. and *Listeria monocytogenes* according to EN ISO 6579-1:2017/A1:2020 and ISO 11290-1:2017, respectively. Microbial groups considered in this research were total mesophilic bacteria (TMB), *Lactobacillus spp*., *Pseudomonas*, sulfite reducing anaerobic bacteria, total Coliforms, *E. coli*, and coagulase positive staphylococci.

^{1}H-NMR analysis

A ^{1}H-NMR analysis solution was prepared, constituted by a 10 mM D2O solution of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) as a chemical-shift reference (δ -0.017). A 1 M phosphate buffer granted a pH of 7.00 ± 0.02, while 10 µl of NaN_3 (2 Mm) avoided microbial proliferation.
By modifying the procedure set up by Ciampa et al (2012). A trichloroacetic acid (TCA) extraction was performed, by adding 0.5 g of fish muscle to 3 mL of 7% (w/w) TCA, followed by homogenization by Ultra-Turrax (IKA, Germany) at 14,000 rpm for 20 s. The homogenate was centrifuged at 18630 g for 10 min at 4 °C, then 0.7 ml of supernatant was added with 0.100 ml of a D2O solution of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) 10 mmol/L. The pH was adjusted to 7.00 ± 0.02 using 9 mol/L KOH in an Eppendorf microfuge tube. After centrifuging once more under the above conditions, 0.65 mL of supernatant was transferred to an NMR tube for analysis.

2.2 Effects of a multistrain probiotic on cirrhotic patients

Study Design

Patients were randomized by a hepatologist, other than those who selected the patients, to take either a probiotic (probiotic group) or a placebo (placebo group). Randomization was performed by means of a computer-generated sequence using blocks of four and consecutively numbered opaque sealed envelopes.32 patients were treated for 12 weeks and assessed at baseline and at 12 weeks (end of treatment) for clinical and analytical data, complications of cirrhosis, side effects, adherence, cognitive function, risk of falls, systemic inflammatory response, and biomarkers of intestinal barrier and bacterial translocation. There were no statistical differences

between the 2 groups at baseline.

^{1}H-NMR analysis of serum

Serum samples were prepared for ^{1}H-NMR by thawing and centrifuging 1 mL of each sample for 15 min at 18,630 g and 4°C. 500 µL of supernatant was added to 100 µL of NMR analysis solution. Urine samples were prepared for 1H-NMR by means of thawing and centrifuging them for 15 min at 18,630 g at 4°C. An amount of supernatant equal to 350 µL was added to 350 µL of bi-distilled water and to 200 µL of NMR analysis solution. Finally, each of the obtained samples was centrifuged again at the above conditions just before analysis.

3. Results and Discussion

3.1 Results of HHP treatment on the metabolic profile of seafood products

Effect of HHP on microorganisms of considered seafood

Figure 1 illustrates the results of the microorganism analysis. It is obvious that the application of 600 MPa pressure extended the microbiological shelf life of the seafood under consideration from 7 or 9 days to 30 days. All treated samples exhibited lower viable counts throughout the storage period compared to the untreated samples.

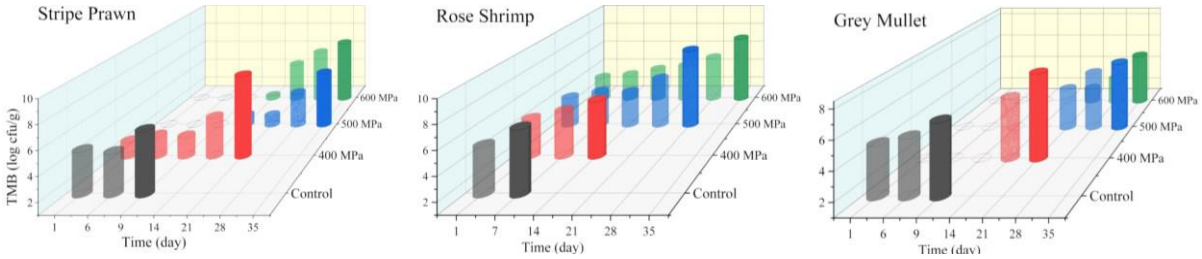

Figure 1 *Changes in microbial cell loads (log CFU/g) of total mesophilic bacteria (TMB) of packaged striped prawn, rose shrimp, and grey mullet during the chilled storage of packaged striped prawn untreated (gray) or after treatment with HHP at 400 (red), 500 (blue) or 600 MPa (green).*

Characterization of seafood metabolome

^{1}H-NMR spectra representative of the samples was shown in the Ph.D. thesis project previously described (Lan, 2022). The spectra of the considered seafood predominantly exhibit metabolite groups such as amino acids, amines, carbohydrates, nucleotides, and organic acids. Notably, there are notable differences in certain molecules between untreated and treated samples. Specifically, signals from specific molecules like putrescine and cadaverine, which are associated with spoilage, were only detected on the final day of storage. Additionally, the intensities of other signals, such as acetate, pyruvate, and threonine, displayed significant variations between treated and untreated samples.

Effect of HHP on freshness-related metabolites during storage

^{1}H-NMR spectra made it possible to quantify the nucleotides used to calculate the freshness of the nucleotide breakdown of the seafood products considered. Based on the distinct nucleotide compositions detected by 1H-NMR in the three seafood (data not shown), Ki, K, and H values were employed to calculate the nucleotide breakdown freshness of rose shrimp, striped shrimp, and grey mullet, respectively. As depicted in Figure 2, the application of HHP treatment clearly exhibited a delay in nucleotide decomposition, effectively retarding the deterioration of freshness. Further analysis of the nucleotide concentrations during storage for each treatment (data not shown) suggests that HHP treatment impeded the conversion of IMP to inosine and/or inosine to hypoxanthine.

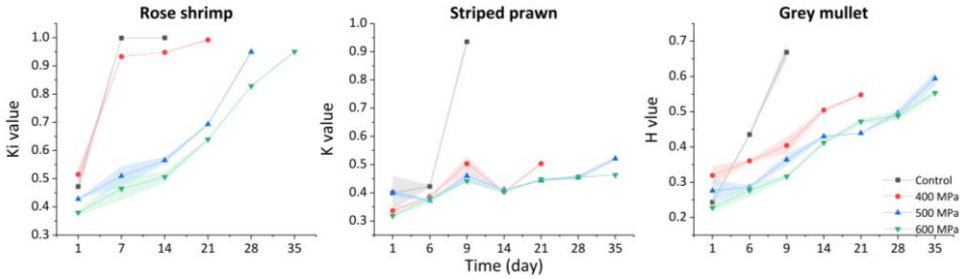

Figure 2 *Changes of nucleotide degradation-related values of rose shrimp, striped prawn, and grey mullet untreated (black squares) and in samples treated with HHP at 400 (red circles), 500 (blue upward triangles), and 600 MPa (purple downward triangles). Ki value is the ratio of the total amount of inosine and hypoxanthine to that of IMP, inosine, and hypoxanthine. K value is the ratio of the total amount of inosine and hypoxanthine to that of all nucleotides. H value is the ratio of hypoxanthine to the total amount of IMP, inosine, and hypoxanthine.*

Effect of HHP on TAV value of sensory active metabolites during storage

To represent the contribution of the concentration of a taste-active compound to the sensory profile, its taste activity value (TAV) was calculated as the ratio of the compound concentration to its taste recognition threshold. In general, compounds with a taste activity value greater than 1 are considered as active compounds in food taste analysis (Figure 3). Figure 3 illustrates the presence of various taste-active compounds in rose shrimp, striped prawn, and grey mullet on day 1. In rose shrimp, glutamate and IMP were detected at concentrations surpassing the threshold for umami taste. Additionally, lysine, glycine, and alanine were identified as significant contributors to the sweet taste profile of rose shrimp. In the case of striped prawn, lactate, IMP, and arginine exceeded the threshold concentrations for sour, umami, and bitter tastes, respectively. Moreover, glycine, lysine, and alanine were found to have concentrations above the threshold for sweet taste in striped prawn. Lastly, in grey mullet, histidine and lactate surpassed their respective thresholds for bitter and sour tastes. Another intriguing observation was both HHP treatment and storage had an impact on the TAV of taste-active compounds. This was observed especially for the TAV of acetate, which tended to be above 1 in three untreated seafood after 7 or 9 days of storage. In contrast, in 500 and 600 MPa treated seafood samples, the concentrations of acetate remained below the threshold after 7 or 9 days. A similar result was observed in concentrations of succinate and of IMP in rose shrimp and striped prawn. Lower concentrations of acetate and succinate and higher concentrations of IMP in treated samples compared with this in untreated samples suggest that HHP can reduce the sour metabolism production of acetate and succinate and slow down the degradation of IMP with umami taste.

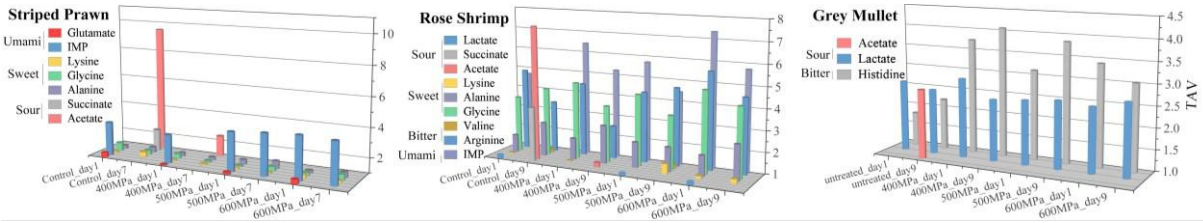

Figure 3 *Taste-active molecules with TAVs greater than 1 in untreated and treated (400, 500, 600 MPa) rose shrimp, striped prawn, and grey mullet on day 1 and day 7 or 9. Not showed represents TAV less than 1.*

3.2 Results of a multistrain probiotic on cirrhotic patients

Characterization of cirrhotic patients' serum metabolome

The ^{1}H-NMR representative spectra of cirrhotic patients' serum were shown in Figure 4. The untargeted analysis of the serum metabolome using 1H-NMR enabled the clear identification of 54 metabolites.

Pairwise comparisons of metabolites in the probiotic group and in the placebo group

Figure 5 showed the pairwise comparisons between values at baseline and at 12 weeks. The probiotic group exhibited a significant increase in glutamine (p=0.002, FDR p=0.007) and a decrease in glutamate (p=0.03, FDR p=0.03), resulting in an elevated glutamine/glutamate ratio (p=0.009, FDR p=0.01). Conversely, the placebo group showed an increase in glutamate concentration (p=0.01, FDR p=0.02) and a decrease in the glutamine/glutamate ratio (p=0.02, FDR p=0.03). No statistically significant alterations were observed in any of the other identified metabolites. These findings suggest that the administration of multispecies probiotics can influence glutamine/glutamate metabolism and enhance the capacity to detoxify ammonia.

4. Conclusions and Future Perspectives

The present research achieved a better understanding of the effect of HHP treatment on the metabolism of seafood. In particular, the application of HHP could effectively extend the microbiological shelf life and delay the degradation of freshness-related and taste-related metabolites resulting in a higher quality associated with freshness and flavor. The great intriguing thing is obtaining information on the main freshness index as well as the molecules umami, sweet, sour, and bitter related in seafood. Moreover, the potential mechanism underlying the effect of a multistrain probiotic on patients with cirrhosis was revealed. The use of probiotics significantly decreased blood glutamate levels and increased the glutamine and glutamine/glutamate ratio. A possible mechanism is the multispecies probiotics influence glutamine/glutamate metabolism and improve the ability to detoxify ammonia.

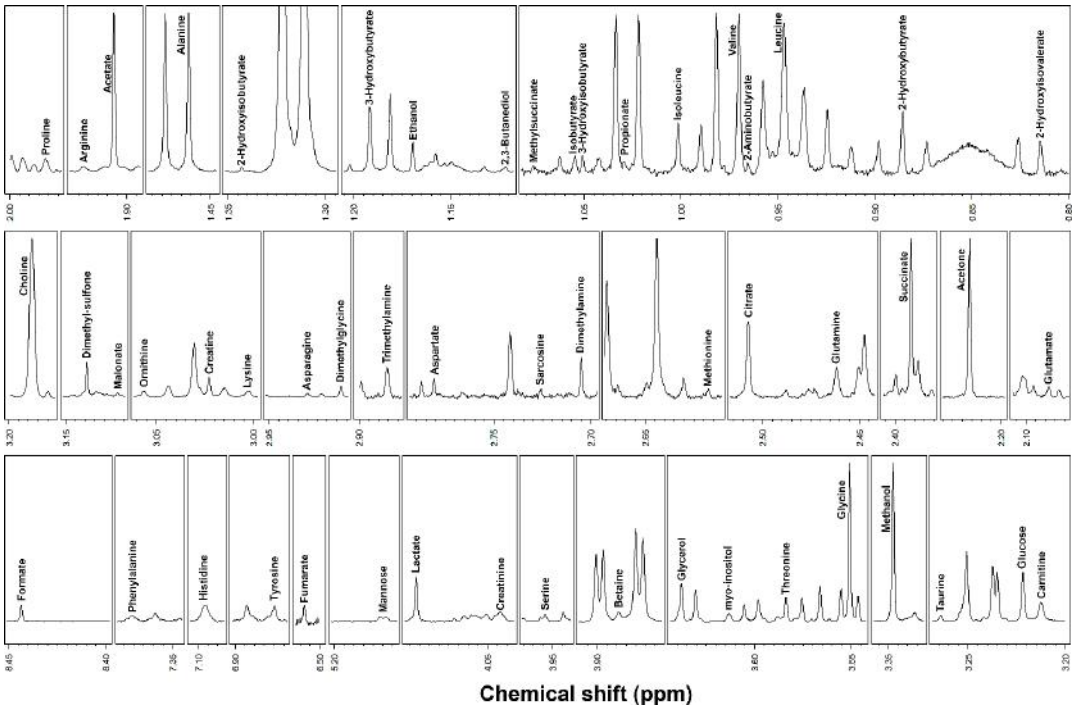

Figure 4 *Samples of 1H-NMR spectra of serum of patients with cirrhosis.*

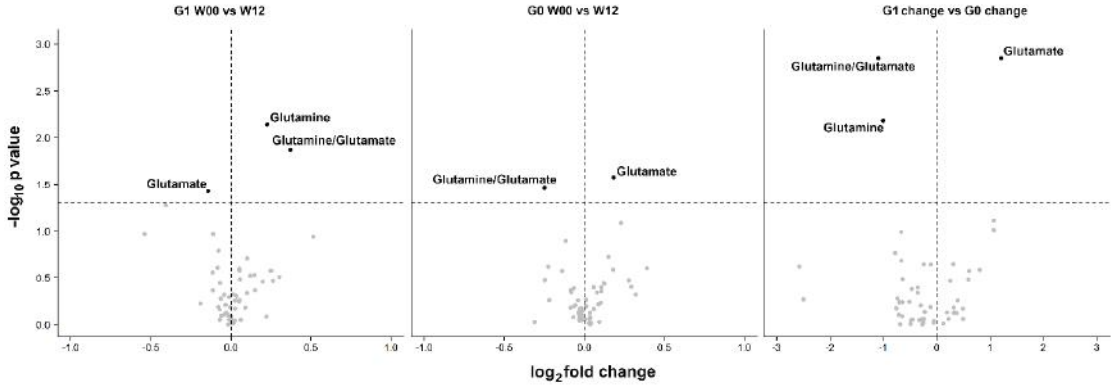

Figure 5 *Volcano plots showing the change between baseline and 12 weeks in all metabolites identified in the probiotic group and in the placebo group.*

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Study and Evaluation of Strategies for Replacing Plastic Materials with Greener and Eco-Sustainable Alternatives

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In this PhD thesis, strategies for the replacement of common plastic materials of non-biodegradable petroleum origin with eco-sustainable and renewable alternatives have been studied and evaluated. We were concerned not only with testing the performance of paper-based packaging materials and renewable materials already on the market, but also and above all, the creation of films and coatings on kraft paper to improve their performance and use them as substitutes for common plastic packaging.

Studio e Valutazione di Strategie per Sostituire i Materiali Plastici con Alternative più Green ed Ecosostenibili

In questa tesi di dottorato sono state studiate e valutate strategie per la sostituzione dei comuni materiali plastici di origine petrolifera non biodegradabili con alternative ecosostenibili e rinnovabili. Ci siamo preoccupati non solo di testare le prestazioni dei materiali di imballaggio a base carta e dei materiali rinnovabili già presenti sul mercato, ma anche e soprattutto, della creazione di film e rivestimenti su carta kraft per migliorarne le prestazioni e utilizzarli come sostituti dei comuni imballaggi in plastica.

Key words: compostable bioplastics; coatings; food-paper; renewable materials; food-packaging.

1. Introduction

In this oral communication the main results of the following activities will be reported
A1) Comparison of the performance of packaging made from environmentally sustainable and renewable materials with non-biodegradable petroleum-based packaging.
A2) Strategy for improvement the performance of paper for food use with the use of biopolymers films made by coating technique.

A1. Replacement of traditional plastic packaging for fruit and vegetables

In the food industry, a growing concern is the availability of packaging materials with suitable thermal, mechanical and barrier characteristics to prevent contamination and food waste, maintaining an adequate shelf life, etc., but greener and more eco-sustainable. To achieve this goal, biopolymers should be affordable, renewable, and available in abundance. In this regard, bioplastic packaging materials based on renewable biomass could be used as a sustainable alternative to petrochemical plastics. The three most commonly used bio-based plastics with unique properties are PLA, Starch based plastics and Cellophane (Tyagi *et al*., 2023). Like for fossil-based plastics, careful selection of a bio-based and/or biodegradable packaging material is necessary to ensure that a packed product has the required shelf life. First all, it is quite important remembering that the terms bio-based and biodegradable are not synonymous; indeed, - 'Bio-based' is defined in European standard EN 16575 as 'derived from biomass' and - Biodegradable materials are materials that can be broken down by microorganisms (bacteria or fungi) into water, naturally occurring gases like carbon dioxide (CO2) and methane (CH4) and biomass (e.g. growth of the microorganism population) (Van den Oever *et al*, 2017) . In this regard, it should be noted that (*Table 1*) not all materials defined as bio-based enter the category of biodegradable materials, and even the petrochemical material can be biodegradable.

Table 1. *Biodegradable and Non-biodegradable materials.*

	PETROCHEMICAL	PARTLY BIO-BASED	BIO-BASED
NON-BIODEGRADABLE	PE, PP, PET, PS, PVC	Bio-PET, PTT	Bio-PE
BIODEGRADABLE	PBAT, PBS(A), PCL	Starch blends	PLA, PHA, Cellophane

A2. Focus on improvement of paper for food-use.

One of the most widely studied materials is paper, a renewable and biodegradable material, mainly composed of cellulose from a wide range of sources in nature, used as primary and secondary food packaging (Oloyede and Lignou, 2021; Deshwal *et al*., 2019).

However, base paper (uncoated paper) is not suitable for food with a long shelf-life, because of its inherent shortcomings, such as poor microbial resistance, low mechanical properties and a porous structure which essentially make it difficult to prevent the penetration of moisture, oils and oxygen. Actually, to overcome these drawbacks, with the aim to expand the application of paper, various advanced functionalization technologies have been extensively studied and developed. For example, paper is commonly coated with chemicals or laminated with aluminum foil or plastic thin films to improve its barrier effect to water vapor, oxygen, mineral oils, and grease (Kopacic *et al*., 2018), but these solutions have some drawbacks such as limited recyclability and compostability. For these reasons, eco-sustainable approaches have to be studied and evaluated and one of the focus points of the PhD work was the formulation of several coating for the improvement of paper for food use.

2. Materials and Methods

Different materials were used for different studies according to the purposes to be pursued and consequently different were the methods applied according to the characteristics of the materials that had to be tested. For this purpose, this session has been divided into two sessions.

A1): Materials: R-PET cup closed with perforated snap-on lid, a tray made of a layer of cardboard in pure virgin bleached cellulose fiber (ECF) and a barrier coating suitable for direct food contact, PLA (polylactic acid) tray closed with snap-on lid, R-PET tray hermetically sealed with a PET film and R-PET tray hermetically sealed with laser-perforated PET film top (with average hole diameter 30 μm); two different cultivar of white grapes (*Melanie* and *Sugar Crips*), small red fruits (blackberries and blueberries) and a fruit salad.
Methods: evaluation of atmospheric variation over time for hermetically sealed packages, weight loss assessment, Brix and acidity measurement, shear force over time of grape samples, microbiological load assessment, sensory analysis and statistical data analysis. All evaluations were performed at different shelf lifetimes of the product, so as to evaluate the variation of the parameters over time, comparing the standard reference packaging with the packages obtained with alternative materials.
A2): Materials: calendared bleached paper, poly(-3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV, NaturePlast®, Ifs, France), polycaprolactone (PCL, Sigma-Aldrich, Mw ~80.000), polyethylene glycol (PEG) 200 (Mw ~190–210; Fluka Analytical), poly (vinyl alcohol) (PVA, Incheon, Korea), glycerol 99.5% (Sigma Aldrich, Germany), potato starch (CAS-No 9005-84-9, PanReac AppliChem ITW Reagents), agar-agar (OXOID, Thermo Fisher), (±)-Epichlorohydrin (ECH), zinc nitrate hexahydrate and melamine (Sigma-Aldrich, MO, USA) and sodium hydroxide (Daejung, South Korea).
Methods: grammage and thickness determination, scanning electron microscopy - energy dispersive spectroscopy analysis (SEM-EDS), measurement of water vapor transmission rate (WVTR), oil and grease resistance, water and oil contact angle measurement, mechanical properties, Fourier-transform infrared spectrometry (FT-IR), thermogravimetric analysis (TGA), mechanical properties tests, antimicrobial activity whit clear zone inhibition test and statistical analysis

3. Results and Discussion

A1. Comparison of the performance of packaging for fruits and vegetables made from environmentally sustainable and renewable materials with non-biodegradable petroleum-based packaging.
The primary objective of this work was to evaluate how the use of packaging made with bio-based and/or biodegradable materials could influence the final quality of the selected fruit samples, compared with the same product stored in commercial packaging. All the trials were carried out in duplicate, and it was evident as regards weight loss, the packages not hermetically sealed recorded more significant weight losses. Excellent results were recorded with the cellulose packaging and sealed with a cellophane lid, both for the measurement of weight loss and for the variation of atmospheric content. Excellent results have been obtained for alternative packaging, also with regard to the microbial load, which for all packages, has remained within the limits of acceptability until the end of the shelf life of the product. Finally, the sensorial evaluation (not trained panelists) demonstrated a high degree of acceptability of the fruit products stored in all the different packages, commercial and alternatives, confirming even more the hypothesis that the packaging materials, to date, used for fruit and vegetable products can be replaced with valid alternatives more eco-sustainable and green.
A2. – Improvement of Paper Resistance against Moisture and Oil by Coating with Poly(-3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) and Polycaprolactone (PCL).
In this work a calendered bleached paper (Advantage MG White High Gloss, Mondi Group, Addlestone, UK) was used as reference paper. Commercial polyethylene-coated paper and fluorinated paper were used as commercial references. Coating paper solutions were prepared dissolving in chloroform one of the two biopolymers (5% w/v of PHBV or PCL) and dissolved in under magnetic stirring at 60 °C for 50 min, and subsequently, at about 75 °C for 10 min. The optimization of PHBV and PCL coating solutions was attempted

by addition of polyethylene glycol (PEG) (at 5%, 10% and 20% on biopolymer dry weight) to improve coating uniformity and spreadability. Paper samples were coated via bar coating with an automatic film applicator.
The SEM analysis (*Figure 1*) of the developed coated samples showed the disappearance of the typical fiber network of paper and allowed to observe a continuous layer of coating. The smoothness of the coating surface was used as an indicator of good biopolymer solubilization.

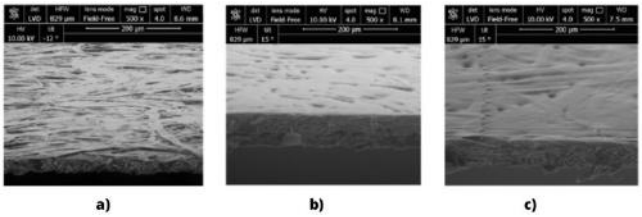


Figure 1*.SEM-analysis of a) Uncoated paper; b) PHBV with PEG10%; c) PCL with PEG 10%.*

Coated samples developed in this work showed a significant improvement of water vapor barrier compared to uncoated paper. The significant reduction of WVTR (*Figure 2*) is a promising feature of developed coated paper samples, according to the importance of this parameter for food quality preservation.

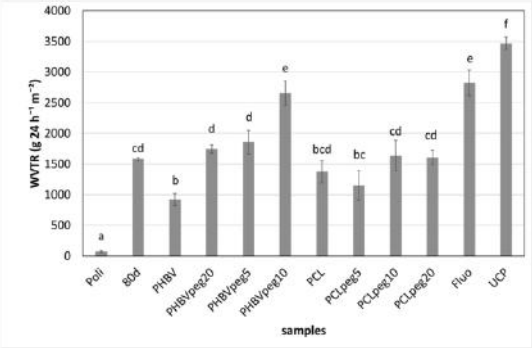


Figure 2. *WVTR values (g 24 h^{-1} m^{-2}) of PHBV and PCL coated samples both pure and with PEG at different concentration, compared to Uncoated paper sample (UCP). "Fluo" refers to fluorinated paper and "Poli" to polyethylene coated paper.*

Coated paper samples showed improved grease resistance, still not comparable with commercial samples; even if, PCL-coated samples showed the best resistance, from 4 to 12 h.
PHBV and PCL coating with PEG at 20% showed good water contact angles. The measured oil contact angles were much lower compared to commercial paper.

- PCL/starch/agar coatings for food-packaging paper: statistical correlation of the formulations' effect on diffusion mechanism and resistance to grease and tensile stress.
In the coating formulation, PCL and glycerol concentration were kept constant, respectively at 5% w/v and 4% (w/v), whereas the amount of agar, starch and PEG was varied among selected ranges.
All the solutions were prepared by dissolving 5% w/v PCL in previously heated ethyl acetate, under continuous stirring in a water bath at 60 °C for 40 minutes. After the complete cooling of the solution, PEG (5% or 15%) was added if required following the experimental plan of the formulations. The water-solutions containing starch (5% or 10%) and the agar-agar (1.5%) in its desired concentrations were prepared separately by stirring at room temperature. Finally, after the addition of the starch-agar solution to the one containing PCL and PEG, a 4% (w/v) of glycerol was added to all the samples. Paper samples were coated via bar coating with an automatic film applicator. From the SEM analysis (*Figure 3*) is evident how the uncoated paper sample showed the normal open and porous network structure with a non-uniform surface, while in all the coated samples the typical cellulose fibers and holes of the paper are not visible.

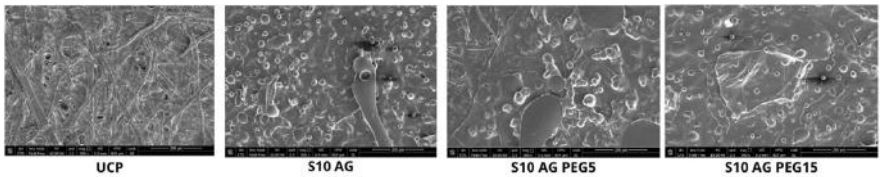


Figure 3. *SEM analysis of UCP) Uncoated paper; S10AG) Sample paper with 5% PCL, 10% Starch and agar; S10AGEG5) Paper sample with 5% PCL, 10% Starch, agar and 5% PEG; S10AGPEG15) Paper sample with 5% PCL, 10% Starch, agar and 15% PEG.*

The addition of starch, even at its lowest level (5%) is fundamental for oil resistance as it has a relevant influence on the contact angle measured with oil. Furthermore, a positive interaction in this sense has been observed when PEG (15%) is employed in the coating formulation, as it leads to positive changes in coating structure. In addition, agar presence has shown in combination with PEG a beneficial key role for oil resistance (*Table 2*, tested by using the standard method, namely T 559 pm-96 and with the contact angle determination) and for water vapor transmission rate, nevertheless, causing a significant detriment of the mechanical properties. The best coating composition has been calculated and it is: 10% Starch, 1.5% Agar and 15% PEG, however, improvements should be made (in terms of new further mixture components) to overcome mechanical properties depletion and to achieve a trend comparable to uncoated paper.

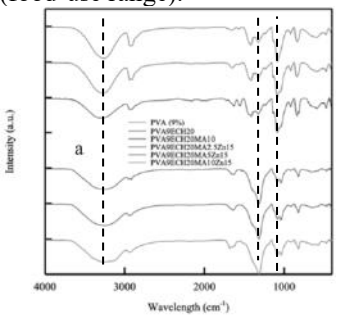
Table 2. Grease resistance values, ANOVA and Tukey's HSD test are reported as FValues and lowercase letter ('a'> 'b' > 'c') respectively, different letters identify significantly different samples ($p \leq 0.05$). * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

	Sample Name		*d.s.*	
S E T 1	**S5 AG**	9.33 ±	0.6	bc
	S10 AG	8.33	0.6	c
	S5	8.67	0.6	bc
	S10	10.3	0.6	ab
S E T 2	**PEG5 S5 AG**	11.3	0.6	a
	PEG5 S10 AG	4.33	0.6	d
	PEG5 S5	8.33	0.6	c
	PEG5 S10	10.3	0.6	ab
S E T 3	**PEG15 S5 AG**	9.33	0.6	bc
	PEG15 S10 AG	8.33	0.6	c
	PEG15 S5	8.67	0.6	bc
	PEG15 S10	9.33	0.6	bc
	UCP	0.33	0.6	e

Multifactorial ANOVA		
AGAR AGAR	STARCH	PEG*AGARAGAR
***	***	*
PEG*STARCH	AGARAGAR*STARCH	PEG*AGARAGAR*STARCH
***	***	***

This study confirms that a well-balanced combination of biopolymers, also from natural origins, could be used to obtain bioplastic coating suitable for the functionalization of paper for food packaging in a circular economy perspective.

- Introduction of the Zn^{2+}-MA complex to polyvinyl alcohol (PVA) as an antimicrobial packaging film.
In this study, the Zn^{2+}–melamine complex was introduced to polyvinyl alcohol (PVA) using epichlorohydrin (ECH) as an epoxide crosslinker; the melamine was used at different concentration to test how changing the formation of the 3D complex and it antimicrobial effect. The interactions between PVA, ECH, MA and Zinc, and the change in the chemical structure of the film were identified via FTIR spectroscopy. Indeed, from the *Figure 4*, we can note the interaction, first all, between the -OH group of PVA with the ECH around 3400 cm^{-1}, then, it is showed the interaction of the Zn^{2+}–MA complex in the PVA film; even the PVA peaks at 1400 cm^{-1} (wagging vibration of -CH) and 1086 cm^{-1} (stretching vibration of C-OH) undergo variations due to the introduction of the Zn^{2+}-melamine complex. The thermal properties of samples, tested by TGA analysis (*Figure 5*), showed that the introduction of the Zn^{2+}–MA complex did not change the film properties in the range between 0 and 200 °C (food-use range).



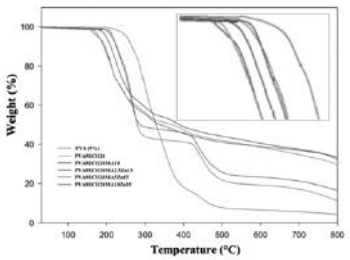


Figure 4. *FT-IR analysis of all samples.* **Figure 5.** *TGA analysis of all samples.*

From SEM-EDS analysis (*Figure 6*) can see the morphology of the samples, the distribution of Melamine (N) and zinc ion and the creation of the 3D structure after the introduction of the zinc ion.

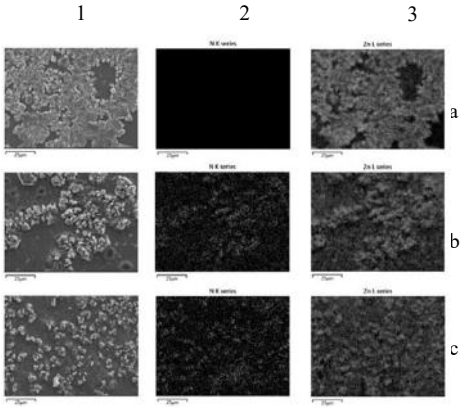


Figure 6. *SEM micrographs illustrating the morphology of* ***a1*** *PVA9ECH20Zn15,* ***b1*** *PVA9ECH20MA2.5Zn15,* ***c1*** *PVA9ECH20MA5Zn15. EDX elemental analysis results showing the distribution of N (****a2*** *PVA9ECH20Zn15,* ***b2*** *PVA9ECH20MA2.5Zn15 and* ***c2*** *PVA9ECH20MA5Zn15) and Zn (****a3*** *PVA9ECH20Zn15,* ***b3*** *PVA9ECH20MA2.5Zn15 and* ***c3*** *PVA9ECH20MA5Zn15).*

Moreover, the antimicrobial properties of the metal-ligand complex (*Table 3*) were evaluated against *S. aureus* and *E. coli* using the zone of inhibition assay. Accordingly, the metal-ligand complex film showed a large inhibition zone against both microbes, in which the zone of inhibition against *E. coli* was bigger compared to *S. aureus.*

Table 3. *Clear zone inhibition test values*

Samples	*E. Coli*	*Staphylococcus Aureus*
PVA 9%	0	0
CTRL1 (PVA9ECH20)	0	0
CTRL2 (PVA9ECH20MA10)	0	0
CTRL3 (PVA9ECH20Zn15)	10±0.5	4.0±1
PVA6E20MA2.5Zn15	13.0±1	5.0±1
PVA6E20MA5Zn15	14.0±1	4.0±1
PVA6E20MA10Zn15	13.0±1	5.0±1
POSITIVE CONTROL	7.5±0.5	6.0±1

4. Conclusions and Future Perspectives

All the studies and the trials done during my PhD thesis period demonstrated that the replacement of common non-biodegradable petroleum-based plastic materials with eco-sustainable and renewable alternatives is possible. The paths to follow are still many and there are still many alternative materials that must be and can be tested.

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Ready-to-Eat Food as a Vehicle of Microorganisms in the Context of the *Microbial Deprivation Hypothesis*

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The aim of this PhD thesis is to investigate whether different cultivation methods can affect the total load and the diversity of food associated microbes. Secondly, this PhD thesis aims to examine if food associated microbes – and especially lactic acid bacteria (LAB) – can survive the human gastrointestinal transit and arrive alive at the intestine.

Prodotti di quarta gamma come veicolo di batteri nel contesto dell'ipotesi dell'igiene

L'obiettivo di questa tesi di dottorato è quello di indagare se diversi metodi di coltivazione possono influenzare la carica totale e la diversità di microrganismi associati agli alimenti. In secondo luogo, questa tesi di dottorato mira a esaminare se i microrganismi associati agli alimenti – in particolare i batteri lattici (LAB) – possono sopravvivere al transito gastrointestinale umano e arrivare vivi nell'intestino.

Key words: Ready-to-eat salad, gut microbiota, microbial diversity, rocket salad.

1. Introduction

In accordance with the PhD thesis project previously described (Mantegazza, 2021), this oral communication reports the main results of the following activities directed to:

A1) Microbial Characterization of commercially available rocket salads, and bacterial strain library set-up;
A2) taxonomic characterization of rocket salad strains;
A3) *in vitro* and *in vivo* survival of lactic acid bacteria associated with rocket salad.

2. Food, bacteria, and diseases

Since the discovery of microorganisms, they have been primarily associated with food spoilage and disease. To combat harmful microbes, various methods for preserving and sanitizing food have been implemented, inadvertently eliminating harmless microbial populations. This unintended consequence of hygiene practices has raised concerns in light of the *microbial depletion hypothesis* (Scudellari, 2017), which suggests that reduced exposure to microorganisms may contribute to immune diseases and allergic disorders. Industrialization and the adoption of modern lifestyles have brought about significant changes in the human gut microbiota. The widespread use of antibiotics, extensive sanitation, and the prevalence of processed foods have led to a reconfiguration of microbial ecosystems, resulting in the rise of chronic metabolic and immune diseases (Blaser, 2016; Sonnenburg & Sonnenburg, 2019). Recent studies have shown that dietary factors commonly found in Western-style diets directly influence the structure of the gut microbiota and promote detrimental metabolic consequences. High consumption of dietary fat, simple sugars, sodium chloride, and synthetic or natural additives alters the composition and function of the microbiota, increases gut permeability, triggers inflammation, and contributes to metabolic dysregulation (Zmora et al., 2019). The role of diet in altering the gut microbiota has led to the suggestion that integrating Western-style diets with fermented foods could counteract the negative consequences of microbial deprivation. Fermented foods, rich in lactic acid bacteria (LAB), have been recognized for their health benefits (De Filippis et al., 2020). However, unfermented raw foods, such as raw vegetables, can also provide a wider taxonomic representation of microorganisms. Raw vegetables introduce microbes from their leaf microbiota and the soil into our gastrointestinal tract, potentially enhancing gut microbial diversity and immune health. Overall, this research aims to shed light on the potential contribution of raw vegetables to the gut microbiota and its implications for human health. By understanding the role of different food sources in shaping the microbiota, we can develop strategies to promote a balanced immune system and mitigate the risk of metabolic and immune diseases.

3. Materials and Methods

In this study, rocket salad samples were analysed to assess the microbial composition and survival during digestion. Commercial rocket salad products and cultivars produced through different farming methods were collected from local retailers. DNA from rocket leaves was extracted using the Qiagen PowerLyser PowerSoil

kit. The V3-V4 regions of the 16S rRNA gene regions were then sequenced through Illumina MiSeq. The counts of mesophilic bacteria and lactic acid bacteria (LAB) were determined using Plate Count Agar or MRS agar at pH 5.7. LAB were isolated and taxonomically characterized using sequencing of 16S rRNA gene amplicons. Simulated gastrointestinal digestion was performed to evaluate the survival of rocket salad-associated microbes during transit through the gastrointestinal tract. To better understand if food associated microbes can survive the human gastrointestinal transit, we performed two interventional trials administering to healthy volunteers 100g of rocket salad (ready-to-eat or extensively washed wish sodium hypochlorite) for three days. Statistical analysis was conducted to compare bacterial counts, assess microbial diversity, and analyse taxonomic compositions between sample groups. The R programming language and GraphPad Prism software were used for the analysis. The unpaired t-test, α-diversity metrics, UniFrac algorithms, and the LEfSe algorithm were employed for statistical analysis and identification of significant differences in microbial compositions.

4. Results and Discussion

4.1 Taxonomic profiling of rocket salad-associated bacteria

The 16S rRNA gene profiling through Illumina MiSeq technology resulted in 4,363,400 reads (mean ± SD, 69,260 ± 21,252 reads). After processing and denoising, 1,024,011 (16,254 ± 4,942) merged reads were obtained. Chloroplast and mitochondrial sequences were removed, leaving an average of 2,984 cleaned reads per sample. Four α-diversity indices were used to evaluate bacterial taxa within each sample. Pielou's, Shannon's entropy, and observed-feature indices showed significantly lower values in vertical farming salads compared to traditional farming salads ($P < 0.001$). Faith's phylogenetic diversity (PD) index was higher in vertical farming salads. No significant differences were observed among traditional farming salads. This analysis indicated that vertical farming salads had reduced taxonomic richness, uneven distribution, and wider phylogenetic distance of bacterial taxa. β-diversity analysis using the weighted UniFrac algorithm revealed notable disparities in bacterial community structures between vertical and traditional farming salads. Principal-coordinate analysis (PCoA) plots showed higher intersample diversity among vertically farmed salads. The different types of traditional farming salads had lower intersample diversity and could not be distinguished based on bacterial community structures. The analysis of bacterial abundances showed distinct patterns between vertical and traditional farming samples. *Eubacteriales*, *Bacteroidales*, and *Lactobacillales* dominated vertical farming salads, while *Pseudomonadales*, *Burkholderiales*, *Flavobacteriales*, and *Actinomycetales* were predominant in traditional farming salads. At the genus level, *Eubacteriales*, *Lactobacillus*, and *Bacillus* were most abundant in vertical farming samples, whereas *Pseudomonas* and *Flavobacterium* were dominant in traditional farming samples. Comparing conventional and organic/integrated farming samples using the LEfSe algorithm, we identified 26 bacterial taxa significantly more abundant in organic rocket salads and 22 taxa overrepresented in conventional salads. Organic farming samples had higher abundances of *Flavobacterium*, *Streptococcaceae*, *Ruminococcaceae*, and *Sutterella*, while conventional farming salads had higher abundances of *Dermabacteraceae*, *Micrococcaceae*, and *Rhodobacteraceae*. Organic/integrated farming samples had different amplicon sequence variants (ASVs) assigned to the genus *Pseudomonas* compared to conventional farming samples. Overall, the analysis indicated that the microbiota associated with vertical farming rocket salads had different bacterial community structures compared to traditional farming salads. Farming practices may impact the taxonomic composition of bacterial communities in rocket salads.

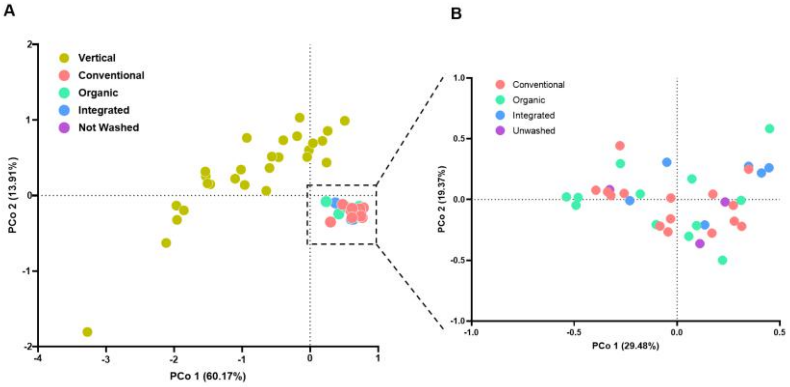

Figure 1 *Inter sample (β -) diversity of the microbiota in mouse intestinal sites shown as principal coordinates analysis of Weighted UniFrac distances based on amplicon sequence variants (ASVs) abundances. A, analysis performed with all investigated rocket salad samples; B, analysis performed with rocket samples from traditional farming only. The first two coordinates (PoC1 and PCo2) are displayed with the percentage of explained variance in brackets.*

4.2 Viable counts of rocket salad-associated bacteria

The results of agar plate count experiments showed that rocket salads grown through vertical farming had significantly higher levels of bacteria compared to rocket salads from traditional farming. This was observed by counting the number of colonies on plate count agar (PCA) and de Man-Rogosa-Sharpe (MRS) media. The mean number of colonies per gram for PCA was 7.41 ± 0.60 CFU/g for traditional farming and 4.77 ± 0.59 CFU/g for vertical farming ($P < 0.001$). For MRS media, it was 1.88 ± 1.24 CFU/g for traditional farming and 0 CFU/g for vertical farming ($P < 0.05$) (Fig. 1). When comparing different traditional farming methods, no significant differences were found in viable cell counts on PCA. The counts were 7.41 ± 0.56 CFU/g for conventional farming, 7.18 ± 0.67 CFU/g for organic farming, 7.70 ± 0.29 CFU/g for integrated farming, and 7.74 ± 0.86 CFU/g for unwashed rocket salads. In terms of lactic acid bacteria (LAB) counts, unwashed rocket salads had more colonies (3.37 ± 0.41 CFU/g) compared to conventional farming (1.61 ± 1.08 CFU/g [$P < 0.05$]) and integrated farming (1.08 ± 1.22 versus 3.37 ± 0.41 CFU/g [$P < 0.05$]), but not organic farming (2.20 ± 1.03 CFU/g [$P = 0.16$]). Overall, the viable cell counts indicated that rocket salads from vertical farming had a significantly lower bacterial load and no detectable viable LAB. Additionally, unwashed rocket salads from conventional farming had a bacterial load comparable to that of ready-to-eat rocket salads from traditional farming, but they had significantly more LAB.

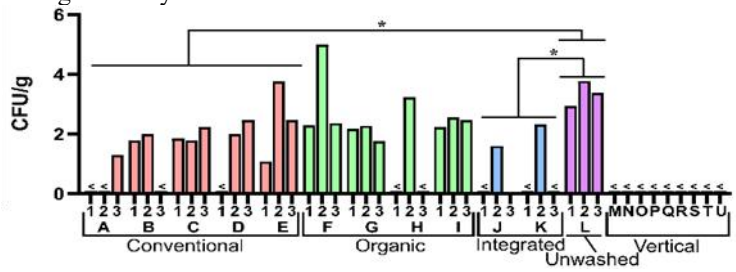

Figure 2 *Viable cell count of bacteria associated to rocket salads as determined on de Man, Rogosa and Sharpe (MRS) agar, expressed as number of colony formant units (CFU) per g of salad. <, under detection limit (10 CFUs per gram of rocket salad). Statistics according to unpaired Student t test; *, P<0.05.*

4.3 Taxonomic characterization of lactic acid bacteria isolated from rocket salad

To determine the distribution of viable LAB in rocket salad, we isolated 237 colonies from MRS agar plates. These colonies were obtained from different types of rocket salads: conventional (95 colonies), organic (84 colonies), integrated (18 colonies), and unwashed (43 colonies). By sequencing the 16S rRNA gene of each isolate, we found that all of them belonged to LAB species, except for two isolates from an organic farming salad that were identified as *Herbaspirillum huttiense*, a Proteobacteria species. The most common genus among isolates from conventional, organic, and unwashed rocket salads was *Leuconostoc*, accounting for 76%, 59%, and 49% of the isolates, respectively. In contrast, *Levilactobacillus* and *Weissella* were the predominant genera in isolates from integrated farming rocket samples. Other less frequent genera included *Latilactobacillus*, *Lactococcus*, *Lactiplantibacillus*, and *Paucilactobacillus*. Overall, we identified 18 different LAB species among the isolates, but none of them were present in all samples. Only four species were found in all types of salads: *Latilactobacillus sakei*, *Leuconostoc mesenteroides*, *Leuconostoc miyukkimchii*, and *Weissella soli*. Principal-component analysis revealed that the presence of certain LAB species could distinguish conventional salads from the others. *Latilactobacillus graminis*, *Leuconostoc citreum*, *Leuconostoc holzapfelii*, *Paucilactobacillus oligofermentans*, and *Paucilactobacillus nenjiangensis* were characteristic of conventional salads, while *Leuconostoc rapi* and *L. carnosus* were associated with organic salads. Integrated salads were characterized by *Levilactobacillus brevis* and *Weissella oryzae*, and unwashed salads were associated with *Weissella koreensis* and *W. cibaria*. The majority of the isolates (96%) were LAB taxa, with *Leuconostoc* being the most prevalent genus (60% of isolates). The distribution of LAB species varied among salad samples and could be partially attributed to different farming practices. Among the isolates, *Leuconostoc mesenteroides*, which accounted for over 20% of all LAB isolates, has demonstrated probiotic activities both in vitro and in vivo. *Weissella* species, constituting about 14% of the isolates, have also shown promising health-promoting activities, such as treating halitosis in the oral cavity. Other LAB species isolated in this study, including *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Latilactobacillus sakei*, and *Levilactobacillus brevis*, are recognized for their probiotic capabilities and ability to survive gastrointestinal transit. Importantly, a simulated digestion experiment indicated that rocket-associated LAB had a significantly higher survival rate compared to other members of the gut microbiota. This suggests that LAB consumed with rocket salad can reach the human gut alive and potentially contribute to microbiome activities, influencing host health.

4.4 Effect of *in vitro* gastrointestinal transit on rocket salad associated LAB

In order to evaluate the potential of bacteria found in rocket salads to survive the journey through the digestive system, we conducted a study where we measured the number of viable bacteria in rocket samples before and after simulating digestion using the INFOGEST protocol (Brodkorb et al., 2019). To quantify the viable bacteria associated with two types of commercially available ready-to-eat rocket salads produced through conventional and organic farming, we utilized PCA and MRS agar following ISO protocols. The simulated digestion process led to a significant decrease in viable bacteria in both conventional and organic rocket salads when assessed using PCA. However, the number of viable LAB quantified using MRS agar was less affected by the simulated digestion process. We also performed the same protocol on two LABs isolated from rocket salad: *Weissella cibaria* and *Leuconostoc lactis*, with or without washed rocket salad. When inoculated together with the rocket salad, the two bacteria showed a survival rate [17.93 ±2 .06% ($p = 0.11$) and 12.47 ± 6.59% ($p = 0.051$) respectively] when compared with the same bacteria without rocket salad [0.003 ± 0.0018% ($p = 0.033$); 0.002 ± 0.00002% ($p = 0.0007$), respectively]. Overall, our findings demonstrate that various taxonomically distinct bacteria associated with ready-to-eat rocket salad have the ability to survive the digestive transit, with LAB exhibiting a particularly enhanced survival capability. Additionally, we can speculate that rocket salad has a protective effect on the bacteria naturally associated with it, allowing them to arrive alive to the human intestine.

4.5 Impact of rocket salads on faecal bacterial communities

Metataxonomics based on 16S rRNA gene profiling was employed to examine the bacterial community structure of faecal samples collected before (F1) and after (F2) a three-day rocket administration phase. The analysis of α-diversity did not show significant changes in the richness or evenness of the faecal bacterial communities following the short-term consumption of rocket. However, the analysis of β-diversity using weighted and unweighted UniFrac algorithms revealed a slight shift in the bacterial community structure for each individual subject across different time points. These changes were smaller compared to the variability observed between subjects and could not be directly attributed to rocket consumption or washing. Next, we conducted a statistical analysis of the metataxonomic data to investigate the influence of rocket consumption and washing on the abundance of individual bacterial taxa. We performed three levels of analysis: (i) a comparison of all faecal samples collected before and after rocket consumption, regardless of washing or rocket type; (ii) a separate comparison of faecal samples collected before and after rocket consumption for each intervention, independent of washing; (iii) a comparison of faecal samples collected before and after rocket consumption, differentiating between individuals who consumed unwashed or washed rocket, and conducted separately for each intervention. The initial analysis revealed modifications in 13 bacterial taxa, with 10 of them belonging to the Clostridia class. The taxa that exhibited an increase included *Acinetobacter schindleri*, an unidentified species from the family *Christensenellaceae*, *Flavonifractor*, and *Methanobrevibacter*. When analysing the data separately for the two rocket interventions, a larger number of significantly altered bacterial taxa was observed. For R1, 17 taxa were identified, and for R2, 26 taxa were identified. In both interventions, the majority of the modified taxa belonged to the Clostridia class. However, the only alteration common to both interventions was the reduction of an unidentified species belonging to the genus *Roseburia*. Finally, the statistical analysis of bacterial abundances for each intervention, considering washed and unwashed rocket consumption, showed significant modifications. For R1, 7 bacterial taxa changed after consumption of unwashed rocket, and 38 after consumption of washed rocket. For R2, 18 and 14 significantly changed bacterial taxa were identified for unwashed and washed rocket, respectively. There were no common changes between the two interventions with unwashed rocket or between those with washed rocket, except for a decrease in the genus *Roseburia* after consumption of washed rocket in both interventions. Overall, the metataxonomic analysis did not show any significant changes in the bacterial community structure that could be attributed to the rocket consumption treatments. Although no associated lactic acid bacteria (LAB) were found in washed rocket salad, there was no significant difference in LAB quantity in the faeces of volunteers after interventions with washed or unwashed rocket salad. This can be explained by the fact that ready-to-eat rocket salad may not provide a sufficient amount of LAB to significantly alter the existing LAB quantity in the human gut. In our study, the LAB quantities in the two rocket salads used were 4.1 log CFU/g for the first intervention (R1 rocket salad) and 2.3 log CFU/g for the second intervention (R2). Even considering a daily intake of 100 g of rocket salad for three consecutive days, the number of LAB cells introduced with rocket salad in our interventions was at least two log units lower than the LAB already present in volunteers' faeces in this study (estimated based on an average amount of 150 g of faeces per defecation).

43.6 Effect of rocket consumption on LAB levels in faecal samples

During the intervention studies, we collected faecal samples to measure the presence of lactic acid bacteria (LAB) by diluting the samples and culturing them on MRS agar medium. The amount of LAB in the faeces varied between 1.0×10^9 and 1.6×10^5 colony-forming units (CFU) per gram, with a median of 1.2×10^7 CFU/g. Interestingly, the viable count of LAB did not show any significant changes in any of the subgroups during the rocket interventions. To further investigate the dominant LAB genera in the rocket salads, namely *Leuconostoc* and *Weissella*, we conducted quantitative PCR assays using specific probes. The DNA extracted from colonies

collected from the MRS agar plates was used as a template for these assays, which provided information about the quantity of viable cells of the targeted bacterial taxa. The results indicated that the number of viable cells of *Leuconostoc* spp. did not significantly change with either unwashed or washed rocket intervention. However, there was a significant increase in *Weissella* spp. following consumption of unwashed rocket but not washed rocket (Fig. 2). This increase was particularly evident in the second faecal sample collected after the end of rocket consumption, but it was no longer apparent in the subsequent sample. Furthermore, the metataxonomic analysis of faecal bacteria after rocket consumption showed no significant changes in the bacterial community structure attributed to the treatments. This result can be attributed to the limited number of participants in the study and the considerable variability of the human gut microbiota among individuals. Although it is known that the composition of the human gut microbiota can change within a few hours, the short duration of our intervention may have influenced the observed results. As we were unable to find any other studies in the scientific literature that assessed the effects of rocket salad on the gut microbiota, it is necessary to conduct further research with larger sample sizes and longer intervention periods to gain a more comprehensive understanding of the potential effects of rocket salad on the human gut microbiota.

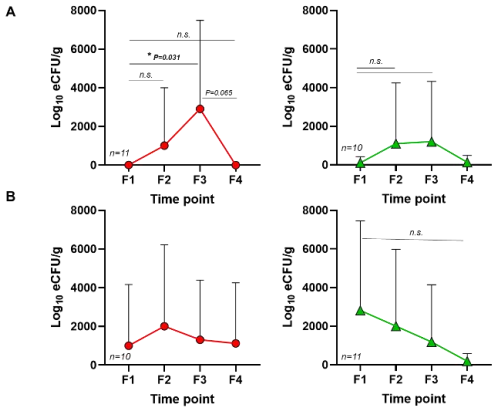

Figure 3 *Estimation of Weissella spp. and Leuconostoc spp. in agar plates of diluted faecal samples. (A) Estimation of Weissella spp. after the consumption of unwashed rocket salad (left) or washed (right). estimation of Leuconostoc spp. after the consumption of unwashed rocket salad (left) or washed (right).*

5. Conclusions and Future Perspectives

Experimental data suggests that modern Western diets, low in naturally occurring microorganisms, may harm health by depleting key microbial taxa in the gut. However, consuming fresh rocket salad from ready-to-eat products could potentially counteract this depletion by providing live bacteria that can survive the digestive system. This hypothesis may apply to all raw plant foods. Further research is needed to confirm the role of raw foods, especially vegetables, in shaping and maintaining the gut microbiota. It should be noted that this PhD thesis focused on fresh-cut commercial rocket salad, and results may vary with different varieties of rocket or other vegetables. The short duration of the study may not fully reveal the benefits of live bacteria in rocket on gut microbiota, necessitating larger-scale and longer-term research. To the best of our knowledge, the interventional study we performed during my PhD, is the first intervention study supporting the idea that raw plant products can serve as a source of live bacteria for the human gut. These findings highlight the importance of investigating the microbial component of raw, non-fermented foods and their potential impact on the human intestinal microbiome.

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Investigation at the boundaries between food processing and nutritional attributes of cereal-based products

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This PhD thesis aims at investigating the effect of transformation processes on the technological, nutritional and biochemical characteristics of cereal-based products. In particular, this thesis has evaluated:

I. the effect of soft wheat milling (roller vs. stone milling) on technological/rheological and nutritional (special emphasis on inflammatory activity) quality of flours;
II. physical-chemical characteristics of acorn and wheat flours, the dough making potential of their mixtures, the effect of acorn flour on bread quality;
III. the impact of sorghum fermentation and fortification with bottle gourd seeds (germinated or not), on technological properties, nutritional value, starch and protein digestibility of sorghum porridge.

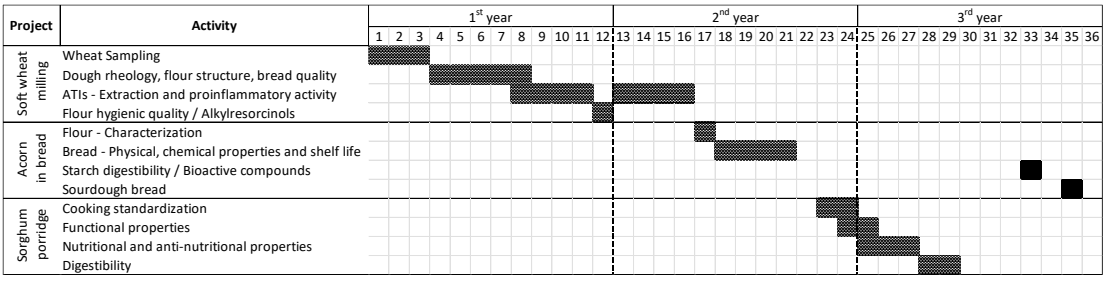
Indagine ai confini tra la trasformazione alimentare e le caratteristiche nutrizionali dei prodotti a base di cereali

Questa tesi indaga l'effetto dei processi di trasformazione sulle caratteristiche tecnologiche, nutrizionali e biochimiche dei prodotti a base di cereali. In particolare, è stato valutato:

1. l'effetto della macinazione (cilindri vs. pietra) del grano tenero sulle qualità tecnologiche/reologiche e nutrizionali (in particolare l'attività infiammatoria) delle farine;
2. le caratteristiche fisico-chimiche delle farine di ghianda e frumento, il potenziale di formazione dell'impasto delle loro miscele e l'effetto sulla qualità del pane;
3. l'impatto della fermentazione e della fortificazione del sorgo con semi di zucca (germinati o meno), sulle proprietà tecnologiche, valore nutrizionale, digeribilità dell'amido e delle proteine del porridge di sorgo.

Key words: cereal-based products, milling, nutritional quality, inflammation, digestibility.

Figure 1 *Gantt diagram representing this PhD thesis. activities done, activities to be done.*



Although all projects will be described in this report, more details will be given on the sorghum porridge project, that was object of the third year research.

I – "Soft wheat milling" project

Even if roller milling (RM) dominates in the food industry (Cappelli et al., 2020), interest has grown in the use of SM, due to the widespread opinion that SM flours have a better nutritional profile than RM flours. As the number of small stone mills has increased, it is important to understand if there is a real difference between flour obtained by these milling systems. Few studies compare the effect of RM and SM on flour quality are present in the literature and the findings could be ascribable to the use of different wheat batches. RM and SM effect was, therefore, studied using a single soft wheat variety (Bologna). RM and SM apply different physical forces (RM generates shear, compression and tearing forces, while SM generates shear, compression and abrasion forces) and temperatures to the flour (higher heat generation in SM), influencing flour properties, in particular protein

stability through oxidative stress. The alteration in the protein stability can affect the technological qualities and the amylase-trypsin-inhibitors (ATIs), that have been associated with non-celiac wheat sensitivity (NCWS) (Volta et al. 2019). The aim of this project was to evaluate how RM and SM affect rheological-structural-nutritional properties, attitude for breadmaking (bread volume, specific volume, texture profile analysis and moisture content), hygienic quality, and ATIs' extractability, oxidative state and, possibly, their pro-inflammatory activity, of whole grain flour (WGF). All flours showed high hygienic quality (mycotoxin below LOD and LOQ). WGF from RM had larger particle size than WGF from SM; more refined flours had smaller particle size. Dough development time, crumb hardness and moisture content were significantly higher in RM WGF, whereas bread volume, specific volume, springiness and resilience were higher in SM WGF. As for flours from the same mill, WGF was significantly higher in dough tenacity/extensibility ratio, water absorption and crumb hardness than more refined flours, whereas it was lower in resilience. Alkylresorcinols content was significantly higher in SM WGF than RM WGF, and inversely related to the refinement degree. On the other hand, although RM and SM of wheat grains resulted in comparable levels of total ATIs, the two milling types differently affected the oxidative state of proteins, with SM flours showing higher levels of 3-nitrotyrosine, dityrosines and carbonyls compared with RM. In turn and interestingly, increased levels in these biomarkers were associated with a higher release of pro-inflammatory cytokines upon treatment of intestinal epithelial cells (Caco-2) with ATIs enriched extracts.

II – "Acorn in bread" project

Acorns are a neglected and sustainable food source that should be given more attention due to its interesting content of bioactive substances (Beltrão Martins, 2020). Application of acorn flour (AF) in leavened products is currently limited. The aim of this research was to examine physical-chemical properties of acorn and wheat flours, the ability of their mixtures to make dough, and the impact of acorn flour (0-50%) on bread quality (volume, texture, and moisture). Acorn flour contained 56% carbohydrates, 6.6% protein, 6.0% fiber and 20.1% fat. Compared to wheat flour (WF), AF showed higher water (69,6%) and oil holding (45,5%) capacity, a higher gelatinization temperature (72°C), and a lower peak viscosity (324 BU, visco amylograph). AF-WF mixtures formed workable doughes up to 20% substitution (Farinograph analysis) with increased water absorption, development time and comparable stability as compared to WF. AF addition to wheat in bread enhanced bread crumb hardness, darkness, moisture content, and decreased specific volume and cohesiveness. AF addition accelerated hardening and retarded cohesiveness loss of bread crumb during storage. Addition of an extra 3% water in bread formulation increased dough development time and stability, resulting in a softer bread with higher specific volume. In the coming weeks the in vitro starch digestibility and the composition of the bio-active substances will be evaluated. The effect of sourdough processing in acorn-enriched bread will also be evaluated.

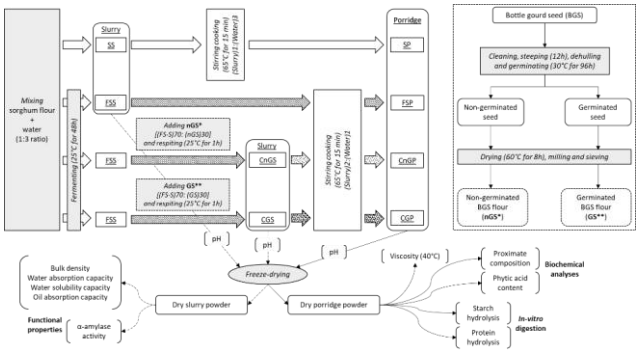
III – "Sorghum porridge" project

1. Introduction

Sorghum porridge is a common homemade complementary food in Africa, but it frequently lacks essential amino acids and is often too thick, making it difficult to swallow and digest for infants 6-12 months (Oladiran and Emmambux, 2020). This project aims at improving sorghum porridge properties for complementary food by means of endogenous sorghum fermentation (to break down phytates and induce textural thinning) and bottle gourd seeds fortification (rich in essential amino acids; also germinated [increase protein digestibility, α-amylase thinning action]). Complementary sorghum porridge was characterised for functional, nutritional/anti-nutritional properties.

2. Methodology

Figure 2 *Samples preparation and analyses. SS: Sorghum slurry, FSS: Fermented SS, CnGS: Composited SS (fermentation + non-germinated seed flour), CGS: Composited SS (fermentation + germinated seed flour).*
SP: Sorghum porridge, FSP: Fermented SP, CnGP: Composited SP (fermentation + non-germinated seed flour), CGP: Composited SP (fermentation + germinated seed flour).



3. Results and Discussion

3.1 Functional properties

Only CnGS significantly reduced the water absorption capacity (WAC) (Table 1). Oil absorption capacity (OAC) was not significantly different among samples.

The extent of water solubility capacity (WSC), of CnGS and CGS suggest high digestibility of food, which is ideal for infant foods. Fermentation and germination significantly reduced the bulk density (BD) of the composited samples for both fermented and blended flour. Germination and fermentation significantly reduced the pH and significantly increased α- amylase activity. It is evident that microbes during fermentation and hydration activation during germination cause an increase in endogenous α-amylases. The partial hydrolysis of carbohydrates during the processes improves energy sources for lactic acid bacteria and subsequent reduction in pH (Chaves-López et al., 2020), representing an efficient way to inhibit food-borne pathogens for the designed infant food.

Table 1 *Functional properties of blended flour samples. SS: Sorghum slurry, FSS: Fermented SS, CnGS: Composited SS (fermentation + non-germinated seed flour), CGS: Composited SS (fermentation + germinated seed flour). WAC: water absorption capacity, WSC: water solubility capacity, OAC: oil absorption capacity, BD: bulk density.*

Slurry	WAC (g/g)	WSC (g/g)	OAC (g/g)	BD (g/ml)	pH	α-amylase activity
SS	5.38 ± 0.75^{b}	3.01 ± 0.20^{a}	1.70 ± 0.04^{a}	0.86 ± 0.03^{b}	6.33 ± 0.03^{c}	0.31 ± 0.15^{a}
FSS	$5.02 \pm 0.17^{a,b}$	4.74 ± 1.83^{b}	1.92 ± 0.11^{a}	0.71 ± 0.02^{a}	3.84 ± 0.07^{a}	3.37 ± 1.33^{b}
CnGS	4.40 ± 0.33^{a}	$5.57\pm0.82^{b,c}$	1.72 ± 0.04^{a}	0.74 ± 0.06^{a}	4.42 ± 0.01^{b}	3.12 ± 0.44^{b}
CGS	$4.94 \pm 0.26^{a,b}$	6.94 ± 0.40^{c}	1.84 ± 0.08^{a}	0.71 ± 0.03^{a}	4.51 ± 0.05^{b}	2.84 ± 0.42^{b}

3.2 Biochemical properties

The combined treatments increased the ash content of the samples (Table 2), which is expected to parallel the mineral contents, which are crucial for infant wellbeing. There was no significant difference for fibre (presence not preferable in complentatry food due to its dietary bulk and indigestibility). The protein content of the combined treatments samples was significantly higher than that of only fermented samples, while the carbohydrate content of only fermentation was higher than the fermentation-germination combination. The reduction in carbohydrates was expected due to the activation of α-amylase content and because they are the main source of nutrients for microorganisms during the glycolysis pathway (Simwaka et al., 2017). The fat content of the combined treatments is higher when compared to fermentation alone. The reduction in crude fat for germination was expected because of the biochemical and physiological changes such as increased lipolytic enzyme activity for hydrolysis of triglycerides to fatty acids and glycerol (Simwaka et al., 2017).

Table 2 *Proximate composition of porridge samples. SP: Sorghum porridge, FSP: Fermented SP, CnGP: Composited SP (fermentation + non-germinated seed flour), CGP: Composited SP (fermentation + germinated seed flour). nGS: non-germinated seed, GS: germinated seed.*

Porridge	Nutritional composition g/100g						Energy kJ/100 g
	Moisture	Fibre	Ash	Protein	Fat	Carbohydrate	
SP	9.43 ± 1.06^{c}	1.92 ± 0.36^{a}	0.25 ± 0.13^{a}	10.63 ± 0.4^{a}	2.04 ± 0.12^{a}	74.37 ± 1.06^{c}	1497.8 ± 20.3^{a}
FSP	6.84 ± 1.52^{b}	1.91 ± 0.12^{a}	0.83 ± 0.99^{a}	9.84 ± 2.2^{a}	2.81 ± 0.85^{a}	76.40 ± 2.15^{c}	1547.6 ± 40.7^{b}
CnGP	4.18 ± 1.40^{a}	1.78 ± 0.19^{a}	1.08 ± 0.19^{a}	17.94 ± 0.96^{b}	14.86 ± 1.01^{c}	58.66 ± 1.35^{a}	1839.6 ± 44.4^{d}
CGP	3.69 ± 0.19^{a}	2.08 ± 0.75^{a}	1.61 ± 1.30^{a}	18.25 ± 0.82^{b}	10.68 ± 1.21^{b}	62.50 ± 0.74^{b}	1751.7 ± 28.3^{c}
nGS	ND	10.53 ± 1.54^{b}	ND	35.63 ± 0.82^{c}	48.55 ± 2.05^{d}	ND	ND
GS	ND	14.8 ± 2.55^{c}	ND	35.18 ± 2.15^{c}	36.69 ± 0.82^{e}	ND	ND

The combined treatments allowed to achieve an increase in energy content, while CnGP showed higher energy density than CGP corresponding to the fat content of the same. In addition to that, CnGP had higher α-amylase which hydrolyses the amylose and amylopectin to dextrins and maltose, thus causing the simultaneous increase in energy density. The energy content of all the porridges fell within the recommended values for children 9-11 months for a once-a-day consumption. Feeding frequency is also important in determining the extent to which energy and nutrient requirements are met. The calculated estimates for three-times-a-day consumption (Table 3) point that a higher frequency of servings of the porridge blend would meet the RNI for children in the weaning age groups. This suggested that bottle gourd seed flour could be added to increase energy content for 6-11 months children, and to increase protein and fat content for 6-24 months children.

Table 3 *Estimated protein and energy intakes of infants (6-24 months) of porridge samples. SP: Sorghum porridge, FSP: Fermented SP, CnGP: Composited SP (fermentation + non-germinated seed flour), CGP: Composited SP (fermentation + germinated seed flour). BME: Breast milk energy, RDI: Recommended daily intake.*

Porridge	Energy (kJ/100g)	Energy intake (kJ/day)			Protein intake (g/day)		
		6-8 months	9-11 months	12-24 months	6-8 months	9-11 months	12-24 months
SP	1497.8±20.3[a]	1865.1	2134.8	2584.2	13.24	15.15	18.35
FSP	1547.6±40.7[b]	1927.2	2205.8	2670.2	12.25	14.02	16.97
CnGP	1839.6±44.4[d]	2290.8	2622.0	3173.9	22.34	25.57	30.96
CGP	1751.7±28.3[c]	2181.4	2496.7	3022.4	22.72	26.01	31.48
RDI Average BME		1490.0	2004.0	3230.0	2.00	3.10	5.00

3.3 Functional properties - Viscosity

Viscosity of the blended porridge samples is shown in Figure 3A. Viscosity decreased in the order FSP> CnGP> CGP~SP. The viscosity of all samples decreased with increasing shear rate (0.1-100s-1) indicating that the porridges exhibited pseudo-plastic behaviour also known as shear thinning behaviour. Typically, during fermentation and germination, amylose and amylopectin are hydrolysed, resulting in significant absorption of water and reduced viscosity when preparing porridge. The reduced viscosity increases the nutrient density in weaning foods (Chaves-López et al., 2020). However, the current formulation has an inappropriate viscosity for children 6-9 month. The limited decrease in viscosity is suitable for children 10 months and older.

3.4 Biochemical properties - Phytic acid

The phytic acid content of fermented-germinated porridge blends is presented in Figure 3B. The trend in lowered phytic acid contents was in the order CnGP > CGP > SP> FSP. The phytic acid content was higher when bottle gourd seed was blended in the porridge. It is noted that the phytic acid decreased with germination and fermentation, confirming that these processes cause significant phytic acid reduction. The reduction in phytic acid is crucial as it enables the adequate availability of protein and minerals which are of paramount importance especially when designing complementary foods (CFs) that can mitigate the malnutrition challenges. However careful consideration should be given if the reduction is not also a result of insoluble complexes between phytate and other nutrients such as phytate-protein-mineral (Siddhuraju and Becker, 2001), which can have a negative effect on the CFs design.

3.5 In-vitro digestion

Starch hydrolysis

Figure 3C reports the kinetics of in-vitro starch digestion of porridges. Starch in-vitro digestibility was in the order FSP>SP>CnGP>CGP. All samples showed a digestibility of less than 25% and this could be explained by the presence of polymeric tannins which have an effect on the physical properties of starch influencing its digestibility (Amoako and Awika, 2016).

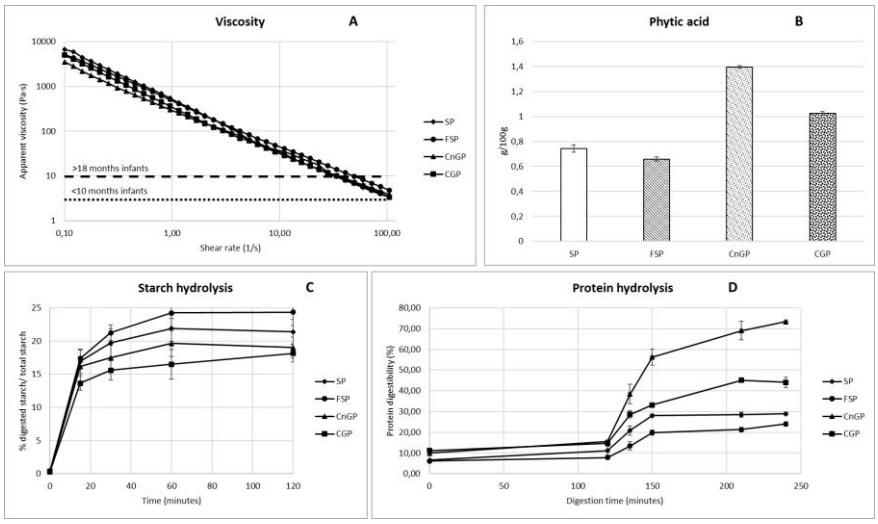
CnGP and CGP had the highest phytic acid content which contributes to the reduction in digestibility of carbohydrates. The fact that fermentation-germination samples had higher protein content can affect the functional properties of starch by influencing the digestibility of both nutrients and also could have been responsible for their lower starch enzyme susceptibility. Protein bodies encapsulating starch granule surfaces can be an extra barrier to the availability of starch to amylase in-vitro thus decreasing digestibility (Rovalino-Córdova et al., 2019). Chlorogenic acid, a phenolic compound identified in BGS, has been reported to inhibit enzyme activity by reducing the number of binding sites for enzymes during starch digestion (Karim et al., 2017).

Protein hydrolysis

The combination of technologies fermentation-germination increased protein digestibility as observed in Figure 3D. In-vitro protein digestibility was in the order CnGP>CGP>SP>FSP. The results that germination treatment did not improve the protein digestibility of the seed flour in the combined treatments were unexpected. Such differences can be attributed to enzyme specificity during digestion, mode of action and conformational state of

proteins (Rovalino-Córdova et al., 2019). The protein digestibility for CGP was higher than SP and FSP, probably due to increased protease hydrolysis activity, and protein solubility because of the hydrolysis of complex storage protein to more soluble amino acids by fermentation-germination and removal of protease inhibitors.
Unblended sorghum samples SP and FSP had the lowest protein digestibility probably due to the interaction of sorghum proteins with non-protein components protein-tannin complexes, enzyme inhibitors or changes in protein structural characteristics (Duodu et al., 2003; Rodríguez-España et al., 2022). Together the combined treatments proved to be an effective processing approach to add nutritional value for CFs.

Figure 3 *Viscosity, phytic acid, starch hydrolysis and protein hydrolysis of blended porridge samples. SP: Sorghum porridge, FSP: Fermented SP, CnGP: Composited SP (fermentation + non-germinated seed flour), CGP: Composited SP (fermentation + germinated seed flour).*



4. Conclusion and Recommendations

The combination of processing technologies, fermentation and germination, improved the functional properties of the flour blend and subsequent nutritional and physical properties consolidating the applicability in infant complementary foods production. Neither fermentation nor germination alone is optimal in improving the target product functionalities of sorghum and bottle gourd seed flour blend. Furthermore, the synergistic effect of "combination processing treatments" and the "effect of ingredient macronutrients on functionality" is a useful tool for the exploration of the design of affordable and diversified new complementary infant foods.
Therefore, before undertaking in-vivo or human studies, further research is recommended on (i). Interrelationships of processes when using highly nutritious underutilized crops from different regions, (ii). A detailed investigation into functionalities of various ingredients and target compounds and (iii). Design of a traditional complementary foods using the combinations, that suit infants below 10 months.

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Application of non-conventional yeasts to improve the quality of innovative tropical fruit beverages

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The present PhD thesis dealt with innovations in technology of production of tropical fruit beverages. In the first part of the PhD programme, ecological niches associated with sugar-rich sources such as manna and fermented honey by-products were investigated. The high sugar content (about 80% w/w) makes these matrices extremely selective for microorganisms with potential food applications. Yeast strains have been isolated, characterized and applied as starter and co-starter cultures in fruit beer production. To this purpose, *Eriobotrya japonica* fruits were used. The improvement of physicochemical and sensory quality of the final product have been evaluated.

Applicazione di lieviti non convenzionali per migliorare la qualità di bevande innovative a base di frutta tropicale

La presente tesi di dottorato si è occupata dello sviluppo tecnologico di bevande innovative a base di frutta tropicale. Nella prima parte del dottorato sono state studiate le nicchie ecologiche associate alle fonti altamente zuccherine rappresentate dalla manna e dai sottoprodotti del miele fermentato. L'elevato contenuto di zuccheri (circa l'80% in peso) rende queste matrici estremamente selettive per i microrganismi con potenziali applicazioni alimentari. I ceppi di lievito sono stati isolati, caratterizzati e applicati come colture starter e co-starter nella produzione di birra alla frutta. A questo scopo sono stati utilizzati i frutti di *Eriobotrya japonica*. È stato valutato il miglioramento della qualità fisico-chimica e sensoriale del prodotto finale.

Key words: Alcoholic fermentation; *Eriobotrya japonica*; non-*Saccharomyces* yeast; *Saccharomyces cerevisiae*; sugar-rich matrix.

1. Introduction

In accordance with the PhD thesis project summarized above, the present oral communication reports the main results of the following five activities:

(A1) *in vitro* evaluation of *Hanseniaspora uvarum* and *Saccharomyces cerevisiae* strains, isolated from sugar-rich matrices, such as honey and manna ash, for producing experimental fruit beers. The strains were selected as a result of a preliminary investigation (e.g. ethanol resistance, H_2S production, hop resistance or consumption of sugars);

(A2) technological screening to evaluate the beer wort fermentation capacity of *Lachancea thermotolerans* strains isolated from manna ash;

(A3) application of *S. cerevisiae* and *L. thermotolerans* strains under medium scale level conditions to several experimental beer productions including addition of loquat, mango and blackberry fruits;

(A4) microbiological analysis and determination of the main physicochemical parameters of samples collected during the different stages of beer production. Finally, volatile organic compounds (VOCs) and sensory analyses of the fruit beers produced were carried out to evaluate the effect of yeast strains applied.

2. Microbiological study on sugar-rich matrices

The increasing interest in novel beer productions focused on non-*Saccharomyces* yeasts in order to pursue their potential in generating groundbreaking sensory profiles. In fact, fermenting yeasts mostly influence beer production. These agents have a direct effect on flavour and quality of the final beers (Larroque et al., 2021). This research focused on the selection of yeasts from sugar-rich matrices with the aim to select new yeast strains capable of producing innovative fermented alcoholic beverages. In particular, the ecological niches associated with highly sugary sources were investigated. In particular, manna, the sugar product obtained from the solidification of processed sap from different *Fraxinus* sp. (Schicchi et al., 2007), and honey by-products used to process a highly alcoholic beverage (Guarcello et al., 2019; Matraxia et al., 2021) were the two sources object of investigation. Both sources, due to the high sugar content, host osmophilic microorganisms. The study resulted in the isolation of several yeast species showing useful characteristics to act as starters or co-starters in food applications such as fruit beer production.

3. Preliminary study and technological screening of selected yeast strains

The yeasts present in fermented honey by-products were identified as *S. cerevisiae*, *Wickerhamomyces anomalus*, *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii* and *H. uvarum* (Matraxia et al., 2021). Manna ash hosted the following species: *Candida aaseri*, *Candida lactis-condensi*, *Citeromyces matritensis*, *L. thermotolerans*, *S. cerevisiae* and *Zygosaccharomyces bailii* (Guarcello et al., 2019) with *L. thermotolerans* being the most represented species. The study was firstly focused on *H. uvarum* YGA 34 and *S. cerevisiae* MN113, isolated from honey by-products and manna ash, respectively. They were applied for preliminary experimental trials after being evaluated and selected for ethanol resistance, H_2S production, hop resistance or consumption of major sugars. Subsequently, fifteen strains of *L. thermotolerans*, all isolated from manna ash, were examined for their ability to produce light sour beer. Interestingly, all *L. thermotolerans* strains showed growth in presence of ethanol and hops and evidenced excellent beer wort fermentation performances. The strain showing the most significant brewing aptitude was selected for further experimental applications.

4. Materials and Methods

4.1 Assays in synthetic beer wort

A craft beer was produced with the addition of loquat (*Eriobotrya japonica* Lindl) juice extracted from local cultivars. The strains *S. cerevisiae* MN113 and *H. uvarum* YGA34 in combination with *S. cerevisiae* MN113 and US-05 strains, were investigated in this study. All trials were fermented in synthetic beer wort. At the end of alcoholic fermentation (AF), an amount of loquat juice was added to the must up to 20 % (v/v) of total volume. A wort medium designed for fermentations was used and prepared according to the wort composition reported by Larroque et al. (2021). Experimental fermented beers were produced at laboratory-scale level (0.75 L sterilised batch). Loquat fruits were harvested at mature stage from a commercial orchard in Sicily. Fruit juice was extracted, filtered and immediately frozen at -20 °C (Tarantino et al., 2021). Loquat juice was added to all trials at the end of AF (day 10) as reported by Gasiński et al. (2020). All trials were inoculated with approximately 2.0×10^6 cells/mL of each yeast strain. The fermentation was carried out at 18 °C under static condition. Planned analysis: microbiological counts; determination of physicochemical parameters (as sugars, main acids and glycerol); determination of VOCs and sensory analysis. The experimental plan included four trials: T1, inoculated with *S. cerevisiae* US-05, used as control; T2, inoculated with *S. cerevisiae* MN113; T3, inoculated sequentially with strain YGA34 and, after 48 h, with strain MN113; T4, inoculated sequentially with strain YGA34 and after 48 h with strain US-05.

4.2 Application of yeast strains in craft beer productions

Unconventional yeasts and loquat juice were added to a wort produced under medium scale level conditions in order to assess the industrial scale-up of the process. Yeast strains used in this study were *S. cerevisiae* MN113 and *L. thermotolerans* MNF105, both isolated from manna ash and selected for their optimal characteristics after preliminary studies. The wort was made using only Pilsen malt (BestMalz, Heidelberg, Germany) to better understand the effect of yeast inoculum. Experimental top-fermented beers were produced at a medium-scale level (5 L batch) using four different inocula. Loquat juice was squeezed from fruits of the white-fleshed local cultivar "Claudia" (*Eriobotrya japonica* Lindl) following the procedure reported at the previous paragraph. As reported by Gasiński et al. (2020), 20 % (v/v) loquat juice was added to all trials at the end of AF (day 10). Samples were collected at different stages of beer production: 0, 3, 6, 10, 11 and 16 days. The fermentation was carried out at 18 °C under static condition. Planned analysis: microbiological counts; determination of physicochemical parameters (as sugars, main acids, glycerol and alcohol); determination of volatile organic compounds and sensory analysis (aroma and taste). Experimental design: TF1, inoculated with *S. cerevisiae* MN113; TF2, inoculated with commercial *S. cerevisiae* US-05, used as a control; TF3, inoculated with *L. thermotolerans* MNF105; TF4, inoculated with commercial strain of *L. thermotolerans* Philly sour (Lallemand brewing), used as a control.

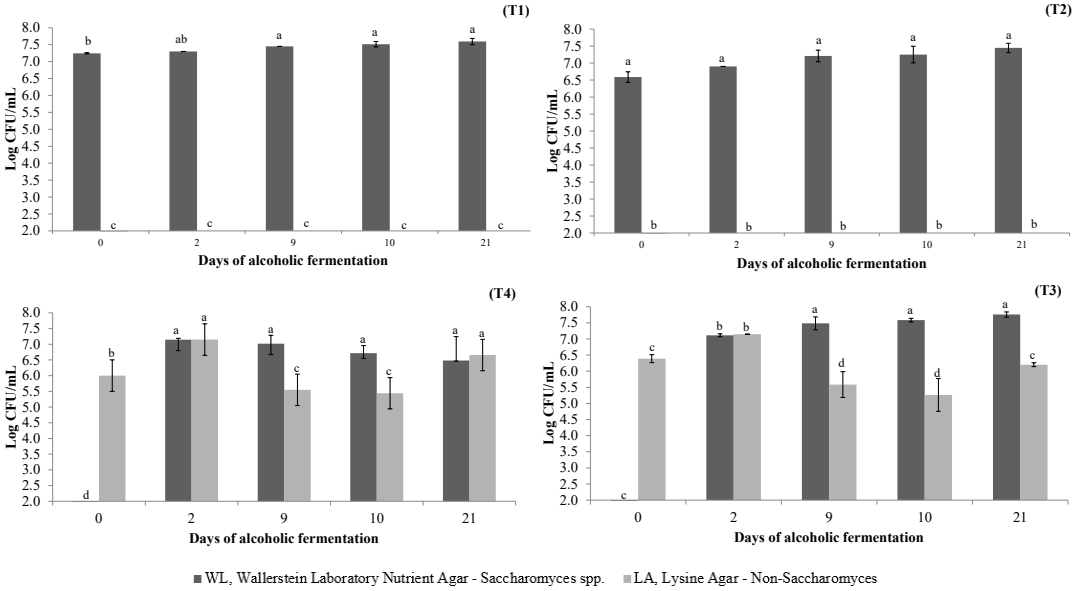
5. Results

5.1 Experimental study with synthetic beer wort

The initial wort had a pH value of 3.00 and 9.70 °Bx, whereas loquat juice was characterized by pH 3.75 and 11 °Bx. The values of pH registered at the end of AF ranged between 3.28 and 3.55. Interestingly, *S. cerevisiae* MN113 in T2 trial showed a more rapid sugar consumption kinetics than control strain *S. cerevisiae* US-05 in T1, including maltose, after 2 days of fermentation. This strain showed an excellent ability to consume sugars in short time. After the sequential inoculum, the concentration of maltose decreased. In fact, at the consecutive sampling day, MN113 together with YGA34 (T3) consumed sugars faster than YGA34 with US-05 (T4). All strains showed their levels in the range 5.0 – 8.0 Log cycles during fermentation. The absence of off-odours and the improvement of aromatic perception were observed in experimental trials involving the use of *S. cerevisiae* MN113 as a monoculture and in sequential inoculum with *H. uvarum* YGA34. Esters and alcohols were the most

abundant compounds emitted from the beers. The beers produced with sequential inoculation of *H. uvarum* YGA34 and *S. cerevisiae* MN113 or US-05 were characterised by higher ester and lower alcohol concentrations. These two unconventional yeast strains, isolated from sugar-rich matrices, showed great technological properties, representing promising co-starters and starter during craft fruit beer production.

Figure 1 *Monitoring of yeast concentrations during AF. Beer fermented by strains: US-05 [T1]; MN113 [T2]; sequential inoculum with YGA34 and MN113 [T3]; sequential inoculum with YGA34 and US-05 [T4]. Different superscript letters indicate significant differences on microbial concentrations were performed at each sampling time according to Tukey's test for P < 0.05. Abbreviations: WL, Wallerstein nutrient agar for yeasts; LA, Lysine Agar for non-Saccharomyces group.*



5.2 Technology screening of *Lachancea thermotolerans* strains from manna ash

The results obtained showed that all strains were able to consume the main sugars present in the wort, low production of hydrogen sulphide. However, but not strains were able to resist to the different levels of ethanol.

Table 1 *Results of fermentation capacity of selected yeast strains.*

	Strain code	H_2S Production[a]	Growth on LA	Growth on WLD	Ethanol tolerance 5/10 % (v/v)	Sugar fermentation		
						Maltose	Glucose	Fructose
Lachancea thermotolerans	MN28	0	+	-	10	+	+	+
	MN136	0	+	-	5	+	+	+
	MN93	0	+	-	10	+	+	+
	MN400	0	+	-	10	+	+	+
	MNF104	0	+	-	5	+	+	+
	MNF105	0	+	-	10	+	+	+
	YS186	0	+	-	5	+	+	+
	YS1	0	+	-	5	+	+	+
	YS42	0	+	-	5	+	+	+
	YS45	0	+	-	5	+	+	+
	YS55	0	+	-	5	+	+	+
	XV11	0	+	-	nd	+	+	+
	XV22	0	+	-	5	+	+	+
	XV34	0	+	-	5	+	+	+
	XV47	0	+	-	10	+	+	+

Symbols: +, positive growth; -, no growth; +/-, weak growth. Abbreviation: H_2S, hydrogen sulfide.
[a]Color of colony on Biggy agar plates: 0 = white; 1 = beige; 2 = light brown; 3 = brown; 4 = dark brown; 5 = black.

Subsequently, yeast strains resistant to 10 % (v/v) ethanol were examined and subjected to further analyses including flocculation, hop and ethanol resistances, fermentation activity (fermentation rate and power, lactic and acetic acid production) during micro-fermentation. The strain MNF105 showed the best technological performances for brewing application.

Table 2 *Results of hop resistance and cross resistance (hop and ethanol) of the yeast strain studied.*

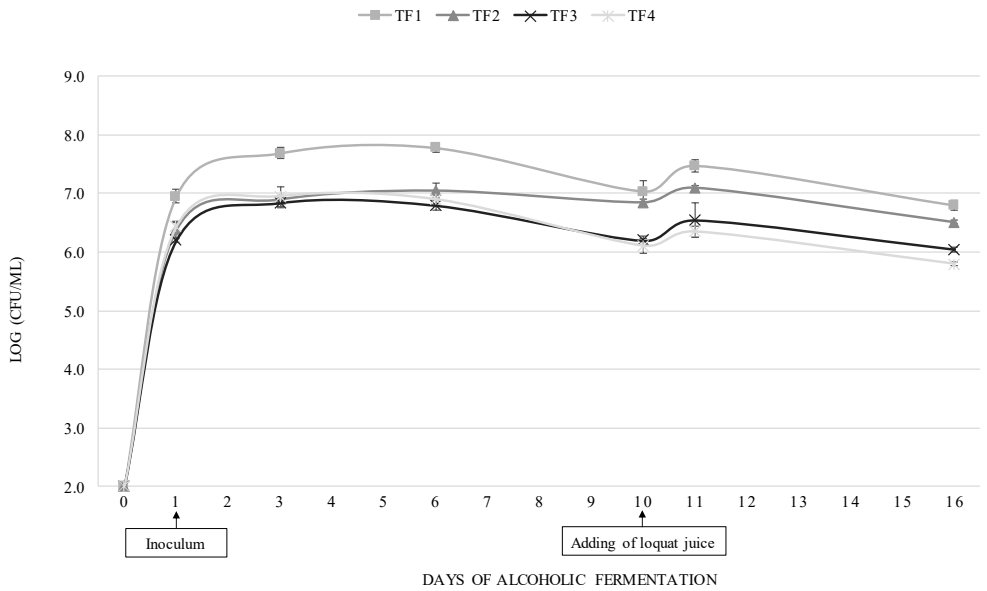
	Hop resistance				Cross resistance (hop and ethanol)			
Strain code	0 IBU	25 IBU	50 IBU	90 IBU	0 IBU/5%	25 IBU/5%	50 IBU/5%	90 IBU/5%
MN28	+	+	+	-	+	+	+	-
MN93	+	+	+	+	+	+	+	+
MN400	+	+	+	-	+	+	+	-
MNF105	+	+	+	+	+	+	+	+
XV47	+	+	+	+	+	+	+	+

Symbols: +, positive growth; -, no growth; +/-, weak growth. Abbreviations: IBU, International Bitterness Unit.

5.3 Application of yeast strains to experimental real beer productions

The initial must showed a pH value of 5.25 and 12 °Bx, whereas the loquat juice was characterized by pH 3.65 and 10.9 °Bx. The pH values recorded at the end of AF were between 3.78 and 3.44 for TF3 and TF4 trials, highlighting the ability of *Lachancea* to acidify the must. As a result, *L. thermotolerans* MNF105 yeast strain showed a low lactic acid production and a marginal influence on the decrease of pH compared to the commercial strain (0.52 g/L and 2.25 g/L respectively). The evolution of yeast populations during the AF is reported in Fig. 2. After inoculation, yeast cell densities ranged between 6.20 and 6.95 Log CFU/mL. The persistence of the strains inoculated was phenotypically investigated by means of colony shape and cellular morphology to recognize typical members of *Lachancea* and *Saccharomyces* genera (Iris et al., 2020). Starter yeast levels increased about 0.5 Log cycles after 3 d for all trials and these results follow the general dynamics of yeast growth in fermenting must-beer. At day 3, trials TF3 and TF4 showed a decrease of presumptive *Lachancea* spp. populations. After loquat juice addition (day 11 of AF), yeast populations levels increased for all trials. At the end of the AF, the highest cell counts were registered for *S. cerevisiae* MN113 in trial TF1 (6.80 Log CFU/mL). Instead, *L. thermotolerans* MNF105 in trial TF3 (6.05 Log CFU/mL) showed values higher than those observed for control trial (5.80 Log CFU/mL).

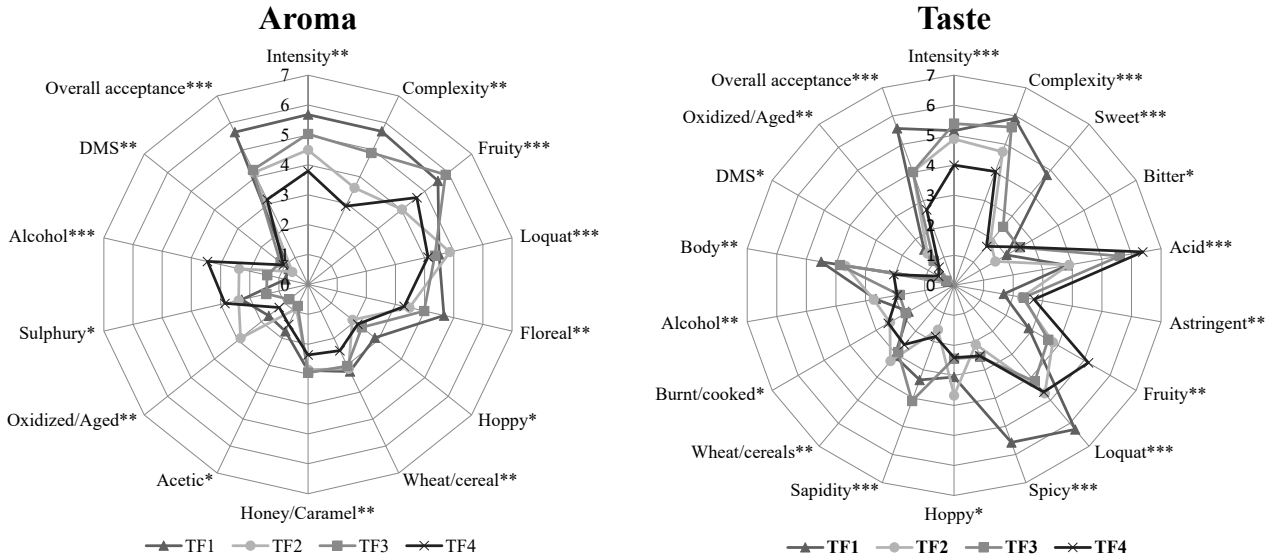
Figure 2 *Monitoring of yeast concentrations during AF. Beer fermented by: S. cerevisiae MN113 (TF1); S. cerevisiae US05 (TF2); L. thermotolerans MNF105 (TF3); L. thermotolerans Philly sour (TF4).*



The overall organoleptic investigation showed a preference for *S. cerevisiae* MN113 (TF1) (values of 5.65 for aroma and 5.56 for taste). Experimental trials conducted with the selected strains demonstrated the absence of off-odour and off-flavour and an improved aroma perception. In addition, beers produced with *L. thermotolerans* MNF105 were more balanced than controls, especially in terms of perceived acidity during sensory analysis (values of 5.60 and 6.38, respectively). This could be due to the lower lactic acid production (0.49 g/L) compared to the control trials (1.74 g/L). Beers produced with *S. cerevisiae* MN113 were characterized by the highest concentrations of alcohols, ketones and carboxylic acids (100.63 ppm, 0.78 ppm and 1.91 ppm). In particular, ethyl acetate, a secondary metabolite of AF responsible for the fruity aroma, was also at high levels (1.27 ppm). Interestingly, the trials inoculated with *Lachancea* strains (TF3 and TF4) showed the highest ethyl

lactate content (0.69 ppm and 2.06 ppm, respectively), a compound produced by this species. These results show that novel yeast strains from unconventional matrices, both *Saccharomyces* and non-*Saccharomyces*, could increase flavour complexity, in agreement with some studies.

Figure 3-4 *Sensory analysis performed on experimental beers: spider plots of average scores for aroma, taste attributes and overall quality of bottled craft fruit beers, determined by judges during tasting sessions. Symbols: ***, P < 0.001; **, P < 0.01; * P <0.05. Abbreviations: DMS, dimethyl sulphide.*



In conclusion, sugar-rich matrices for the selection of yeast starters were explored for the first time and scientific data were provided on their technological feature useful for brewing applications. This work enriches the very limited scientific knowledge on the role of the yeasts *H. uvarum* and *L. thermotolerans* as potential co-starters and starters and also on the effect of loquat fruit as ingredient for brewing. However, further investigations are underway to assess the role of these strains at industrial scale level. Technology transfer trials are in progress at the commercial brewery Epica srl. This research was partially financed by the research project of the Region of Sicily for the support of inland areas.

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Mitigation strategies to reduce food-processing contaminants formation in Neapolitan pizza

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This PhD thesis aimed to evaluate possible mitigation strategies to contain food-processing contaminants formation during Neapolitan pizza production. In particular, the obtainment of a low-asparagine flour for the production of Neapolitan pizza with lower potential to generate acrylamide during baking has been evaluated. Moreover, the effect of different baking process (wood-fired oven and electric oven) on the formation of contaminants such as acrylamide and polycyclic aromatic hydrocarbons was assessed.

Strategie di mitigazione per la riduzione della formazione di contaminanti di processo nella pizza Napoletana

Questa tesi di dottorato ha riguardato la valutazione di possibili strategie di mitigazione della formazione di contaminanti di processo durante la produzione di pizza Napoletana. In particolare, è stata valutata la possibilità di produrre sfarinati con minore contenuto in Asparagina, e quindi con più basso potenziale di formazione di acrilammide durante la cottura di pizza Napoletana, e l'effetto di modifiche del processo produttivo (cottura in forno elettrico e in forno a legna) sulla formazione di contaminanti quali acrilammide ed idrocarburi policiclici aromatici.

Key words: Acrylamide; polycyclic aromatic hydrocarbons (PAHs); Neapolitan pizza; mitigation strategies.

1. Introduction

Following the previous annual report of this PhD thesis project (Quiquero, 2022), the present oral communication illustrates the main results of the following activities:

A) utilization of wheat varieties with low Asparagine (Asn) content for Neapolitan pizza production;
B) production of Neapolitan pizza from low Asn wheat line according to Reg. EU 2017/2158;
C) effect of different baking process (wood-fired oven and electric oven) on the contaminants' formation.

2. Thermal food processing - background

Thermal processes such as frying, baking, and roasting are commonly applied to foods for processing or preservation purposes. They provide the final product with specific flavor, aroma and texture characteristics but toxic compounds can be formed due to the high temperature reached during process, with a deleterious effect on the food quality and safety. The presence of varying amounts of thermal process contaminants in widely consumed foods daily is considered as a major concern by public authorities worldwide; in bakery products the major issue is related not to the level found in food but to the high consumption that make these products a potential risk for human exposure. Therefore, efforts to reduce the amount of these contaminants in heat-treated foods have gained importance (Hamzalioglu 2020; Nerìn et al., 2016). Neapolitan Pizza is one of the most appreciated and popular Italian foods in the world and it has acquired a global significance during last years; in 2010 it has been recognized as a "Traditional specialty guaranteed" (TSG), thanks to EC Regulation 97/2010, and in 2017 received recognition by UNESCO as an "Intangible Cultural Heritage of Humanity". In compliance with the cooking standards provided by the disciplinary of production, Neapolitan pizza is cooked in wood fired ovens with temperature of the dome and bed equal to 430°C and 485°C, respectively, and with a cooking time not exceeding ninety seconds (Falciano et al., 2022). In these conditions, a unique flavor and aroma was provided to pizza, but neo-formed contaminants can be produced. In particular, this study focuses on two food-processing contaminants that can be formed during Neapolitan pizza cooking, acrylamide and polycyclic aromatic hydrocarbons. Acrylamide has been classified as a "probable human carcinogen" (group 2A) with genotoxic and carcinogenic effects. This compound generates during the Maillard reaction from Asn and reducing sugars, naturally present in carbohydrate-rich foods. In 2017, the Commission Regulation 2158 established mitigation measures and benchmark levels to reduce acrylamide content in various food categories; European food safety authority highlighted mean acrylamide concentration in bakery products between 40 and 231 µg/kg, and indicated that ALARA principle should be applied during processing to reduce its levels in the final product, hence reducing consumers exposure. To this aim, food industry and national authorities of the European Union cooperate for the FoodDrinkEurope Acrylamide "Toolbox" as a tool to implement this

regulation through improvement strategies during manufacturing processes. In this research project different crops management strategies were used to produce grains with different Asn content in order to exploit agronomic practices as a potential strategy to mitigate acrylamide content in the final products. The rationale of our experimental approach is that in cereal grain free Asn content has been identified as the main determinant of acrylamide-formation during processing (Raffan 2019). Polycylclic aromatic hydrocarbons (PAHs) are formed and released via the pyrolysis or incomplete combustion of organic materials, and during the industrial processes such as smoking, frying, drying, baking, roasting and charcoal barbecuing (Kaknaz et al., 2019), and can impact both nutritional value and air quality. Research to reduce PAHs contamination and assure food safety and quality in the bakery chain included type of cooking method used, which can have a pronounce impact on PAHs contamination (Singh et al., 2016). To date, no data are available concerning PAHs formation in Neapolitan pizza. In this research project we first assessed the formation of PAHs during traditional cooking of Neapolitan pizza in wood-fired oven; subsequently, the potential formation PHAs was determined using electric oven to assess differences with the traditional approach.

3. Materials and Methods

3.1 Sample obtainment

Wheat samples

Soft wheat of LG Ayrton variety was provided by the University of Torino. Samples consisted of 10 different wheat lots (T2, T6, T7, T11, T12, T13, T15, T17, T19, T20) grown in experimental fields in Piemonte (Italy) during the season 2021/2022. Crop management of different fields was performed by testing different nitrogen dose, sulfur supply, and fertilizer type. Wheat samples have been analyzed for grain characteristics as test weight (kg/hL), performed using a Shopper chondrometer and thousand-kernel weight, TKW (g). Grain samples were milled with a laboratory mill and tested for chemical traits including moisture (ICC method 104/1), protein (AOAC method 992.23), ash (ICC method 104/1) and free Asn content.

Grain milling and flour selection

Aliquots (20kg) of wheat lots were reunited on the basis of their Asn content, then tempered (moisture of 16%) for 16 h to allow an adequate distribution on the surface and milled with a soft-wheat mill (NAMAD SG2000, Rome, Italy), equipped with three break, three reduction rolls and six steel screens, obtaining three breaking rolls flours (B1, B2, B3), three sizing rolls fractions (C1, C2, C3), and bran and shorts. Milling fractions were analyzed for moisture, protein, ash and Asn content. Milling fractions were recombined taking into account Asn content, milling yield, protein, and ash content. Three flours with different Asn content have been obtained: a "00" type flour (F00) with low Asn content by recombining B1, B2, C1, C2 and C3 fractions of the mix low asparagine content (T2, T6, T7, T11), a "0" type flour (F0) by recombining B1, B2, B3, C1, C2 and C3 fractions and a whole-wheat flour (FI) with C1, C2, C3, bran and shorts fractions, from varieties with high asparagine content T15, T19 and T20.

Dough's preparations and cooking conditions

Dough's preparation and samples obtainment were set up according to the traditional manufacturing process of Neapolitan pizza (Commission Regulation EU 97/2010). Pizza doughs were prepared by mixing 1200 mL of water, 2300 g of the selected "00", "0" and "whole-wheat" flour and 2% of sodium chloride. Two different pizza typologies have been obtained, with a topping of tomato sauce and without any topping, both cooked in wood fired oven and in electric oven at 485°C for 90s. Prior analysis, Neapolitan pizza samples were lyophilized using a Virtis Genesis 25ES freeze drying apparatus.

3.2 Chromatographic analysis

Sample extraction and chromatographic conditions for Asn analysis

Asn determination was carried out on soft wheat varieties. The method used for aminoacids extraction was the same illustrated by Curtis et al., 2009, with slight modifications. To 0.5g of finely ground whole-grain flour, 10 ml of 0.01M HCl was added; the sample was stirred for 15 minutes at room temperature and then allowed to stand for a further 15 minutes. An aliquot (1.5 ml) of supernatant was centrifuged at 5000 rpm for 15 minutes. Aliquots of 20 µL of sample were injected into HPAEC-PAD for free Asn analysis after appropriate dilution.

Separation and quantification of free Asn was conducted using an HPAEC-PAD system, model ICS 6000 (Thermoscientific, Milan, Italy). An AminoPac PA-10 column (2x250mm) was employed for chromatographic separation using as eluents H_2O (eluent A), NaOH 250mM (eluent B), acetate 1M (eluent C) with a flow of 0.250 mL/min in a gradient composed as follows: 0-12 min 80%A and 20%B, 12-16 min 68%A and 32%B, 16-40 min 36%A, 24%B and 40%C, 40.1-42.1 min 20%A and 80%B and 42.2-62 min 80%A and 20%B. Using these conditions, asparagine-related peak appeared at 6 minutes and chromatograms were processed using Chromeleon version 7.2.10 (Thermoscientific, Milan, Italy).

Sample extraction for PAHs analysis and chromatographic conditions for PAHs analysis

For PAHs extraction, lyophilized samples were weighted and added with an appropriate quantity of water to reconstitute the sample with the initial moisture value and obtain 5g of wet sample. Acetonitrile-based extraction was performed in a 50mL centrifuge tube mixing the sample with 1 ng/g of spiking standard (PAH calibration mix) and using Tryphenylene (10 ng/mL) as internal standard. After the addition of 5 mL of deionized water and 5 mL of

acetonitrile, samples were vigorously vortexed and left overnight. Afterwards, 2.5 g anhydrous magnesium sulfate and 2.5 g sodium chloride were added, followed by shaking and centrifugation at 4000 rpm for 5 min. Then, 3 mL of the upper layer acetonitrile extract were evaporated to dryness under nitrogen stream at room temperature. The residue was dissolved in 100 µL of acetonitrile and injected into the HPLC-FLD. Gradient elution and fluorescence detection excitation/emission program were set up according to the method described by Viegas et al. 2012. The mobile phase was as follows: solvent A: 75% methanol (in water); solvent B: 100% methanol, solvent C: 100% ethyl acetate with a flow rate 1ml/min. The linear gradient program was: 0–18min, 0–80% B in A; 18–19 min, 80–100% B in A; 19–20 min, 100–90% B in C; 20–28.5 min, 90–82% B in C; 28.5–37.5 min, 82–80% B in C; 37.5–40min, 80–100% B in C, 40–45min 100–0% B in A, rinsing and re-equilibration of column to the initial conditions. Excitation/emission wavelengths selected were 276/330 nm for Na (naphthalene), Ac (acenaphtene) and F (fluorene); 250/336nm for Pa (phenanthrene); 250/402nm for A (anthracene); 270/460nm for Fl (fluoranthene); 270/390 nm for P (pyrene), BaA (benzo[a]anthracene) and Ch (chrysene); 260/430 nm for BbF (benzo[b]fluoranthene); 290/410 nm for BkF (benzo[k]fluoranthene), BaP (benzo[a]pyrene), DhA (dibenzo[a,h]anthracene), and BgP (benzo[g,h,i]perylene); 290/470 nm for IP (indeno[1,2,3-cd]pyrene).

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistic Base (Version 29). Data were subjected to analysis of variance (ANOVA) followed by Tukey's post-hoc test. Results with $p < 0.05$ indicate statistically significant difference. Pearson correlation coefficients were calculated using IBM SPSS Statistic Base (Version 29).

4. Results and Discussion

4.1 Obtainment of flour low in Asn by acting on agronomic practices

Table 1 reports the results concerning chemical composition and Asn content of wheat lines obtained using different cropping practices.

Table 1 *Chemical composition and Asn values of wheat lines obtained from different field management practices.*

	TKW (g)	Test weight (kg/hL)	Moisture (%)	Ash (%d.w.)	Protein* (%d.w.)	Asn (g/kg d.w.)
T2	37,8±0,6abc	83,5±0,0bc	11,5±0,13cd	1,77±0,024ab	11,1±0,02g	0,371±0,006ed
T6	36,8±0,9bc	82,9±0,0d	11,6±0,20bcd	1,75±0,008bc	11,2±0,06g	0,374±0,006ed
T7	39,4±0,9a	83,8±0,0cd	11,7±0,06bcd	1,72±0,008cd	12,2±0,03e	0,355±0,008e
T11	37,5±0,5abc	84,0±0,0a	11,4±0,18d	1,66±0,008d	12,7±0,14d	0,353±0,026e
T12	39,1±0,6ab	83,0±0,5d	11,5±0,10cd	1,71±0,024cd	11,8±0,14f	0,405±0,021ed
T13	38,2±1,1abc	83,2±0,1cd	12,3±0,10a	1,78±0,008ad	12,4±0,02e	0,392±0,022ed
T17	39,4±0,9a	83,1±0,0cd	11,7±0,15bcd	1,81±0,000a	12,3±0,06e	0,427±0,024cd
T15	38,3±0,9abc	83,3±0,0cd	11,7±0,04bcd	1,74±0,020bc	14,6±0,04a	0,493±0,006ab
T19	36±1,0c	83,3 ±0,0cd	11,9±0,07b	1,79±0,016ab	13,6±0,11c	0,470±0,030bc
T20	38,5±1,1ab	84,0±0,0a	11,8±0,15cb	1,75±0,024bc	14,1±0,20b	0,543±0,042a

*Mean values ± sd. Different letters in a column indicate statistically significant differences (p<0.05, T – test). TKW, thousand kernel weight; *N x 5.70;*

The results highlighted that the Asn content of different wheat lines analyzed ranged between 0.353 to 0.543 g/kg d.w., indicating that different agronomic practices are a promising approach to obtain a variable Asn composition of wheat lines (Fig. 1a).

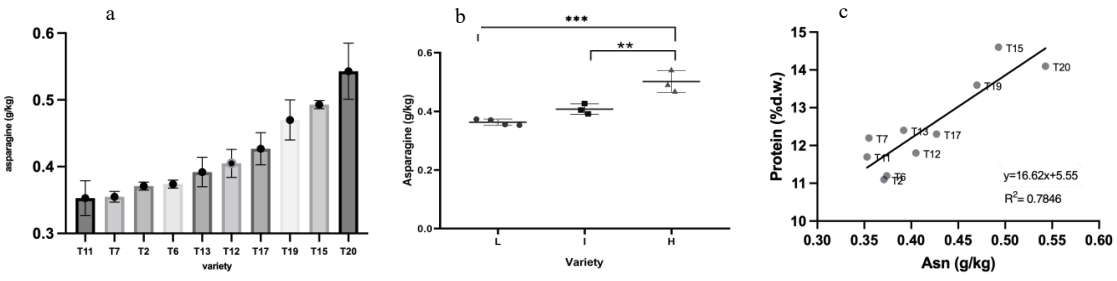

Figure 1. *a) Asn content (g/kg d.w.) of wheat varieties grown using different agronomic conditions; b) Different groups with similar Asn content (g/kg d.w.); c) Correlation between Asn and protein content (% d.w.) of the tested wheat varieties; analysis have been performed through Ordinary one-way ANOVA (Tukey's multiple comparisons test) [**p<0.01; ***p<0.001];*

In particular, a reduction of 54% in Asn is achievable just by choosing the appropriate wheat variety and cropping practices. Although the results demonstrated that it is possible to obtain a variability in Asn

content by acting in the field, environmental factors also have to be considered. Previous studies reported that Asn accumulation is highly influenced by environmental factors such as abiotic and biotic stresses and climatic conditions (Malunga et al., 2019; Lecart et al., 2018; Moustafa et al., 2017). Moreover, results indicates that the average Asn content of lines cultivated with the same apport of sulphur and nitrogen were remarkably similar, resulting in three different groups of wheat varieties according to their free Asn levels ($p < 0.05$), low, intermediate and high Asn content (Fig 1b). In particular, the group with the lowest level of free Asn (mean value 0.363 ± 0.010 g/kg d.w.) included T2, T6, T7, T11; T15, T17 and T19 had the highest content of Asn (mean value 0.502 ± 0.037 g/kg d.w.), while the intermediate group included T12, T13 and T17, with an average value of 0.408 ± 0,018 g/kg d.w. As it concerns the chemical composition, no correlation has been found between Asn content and the other investigated traits, with the exception of a positive correlation ($r = 0.78$, $P < 0.001$) with the protein content, whose values ranged from 11.1 to 14.6% d.w. (Fig. 1c). This result is consistent with previous findings of Lecart (2018), which reported that a strong correlation between Asn and proteins was evident, especially when the protein content of the wheat flour was above 13%. Subsequently, the classes with low and high Asn content were reunited according to their Asn content and milled; in Table 2 chemical composition and Asn content of the obtained milling fraction were reported.

Table 2. *Chemical characteristics of milling fractions obtained from varieties with low and high Asn content.*

	Moisture (%)		Ash (%d.w.)		Protein** (%d.w.)		Asn (g/kg d.w.)	
	low	high	low	high	low	high	low	high
Mix*	**14,0±0,06**	**14,0±0,04**	**1,79±0,03**	**1,79±0,01**	**11,0±0,07 b**	**13,7±0,00a**	**0,389±0,110 b**	**0,575±0,103 a**
B1	15,5±0,08	15,0±0,05	0,64±0,00 b	0,68±0,01a	9,3±0,1b	13,9±0,0a	0,105±0,004b	0,167±0,001a
B2	15,6±0,03 a	14,4±0,12 b	0,72±0,01 b	0,75±0,01a	11,0±0,1b	15,5±0,1a	0,268±0,040b	0,273±0,010a
B3	14,6±0,04 a	13,1±0,00 b	0,91±0,01 b	1,01±0,01a	12,1±0,1b	16,3±0,1a	0,226±0,002b	0,388±0,012a
C1	15,6±0,07 a	14,7±0,07 b	0,56±0,01a	0,52±0,01 b	9,4±0,0b	11,8±0,1a	0,113±0,003b	0,191±0,012a
C2	15,4±0,10 a	14,3±0,07 b	0,63±0,01a	0,55±0,01 b	9,7±0,0b	11,8±0,0a	0,147±0,008	0,178±0,053
C3	15,3±0,12 a	14,3±0,09 b	0,60±0,02a	0,55±0,01 b	9,9±0,1b	11,9±0,0a	0,155±0,009b	0,206±0,010a
Bran	14,9±0,14 a	13,6±0,03 b	5,51±0,01 b	6,04±0,02a	14,6±0,0b	17,5±0,2a	1,145±0,027a	1,062±0,041 b
Short	14,6±0,11 a	13,8±0,01 b	3,68±0,01a	3,29±0,02 b	14,0±0,0b	15,7±0,1a	1,122±0,037a	0,813±0,089 b

*Mean values ± sd. Different letters in a raw (for each parameter) indicate statistically significant differences (p<0.05, T – test). *Obtained with T2, T6, T7 and T11 for low Asn milling and T15, T19 and T20 for high Asn milling; **N x 5.70;*

As showed in figure 2b, the results highlighted a positive correlation between Asn and ash content, indicating a higher content of the Asn in the external parts of kernel. As a consequence, the more a flour is refined, the lower is its Asn content. Three different flours with different acrylamide-forming potential have been obtained through appropriate recombination of milling flours: a "00" type flour, a "0" type flour and a whole wheat flour with Asn content of 0.132, 0.202 g/kg d.w. from the milling of low Asn content group, and 0.363 g/kg d.w. from milling fractions obtained from kernel with high Asn content.

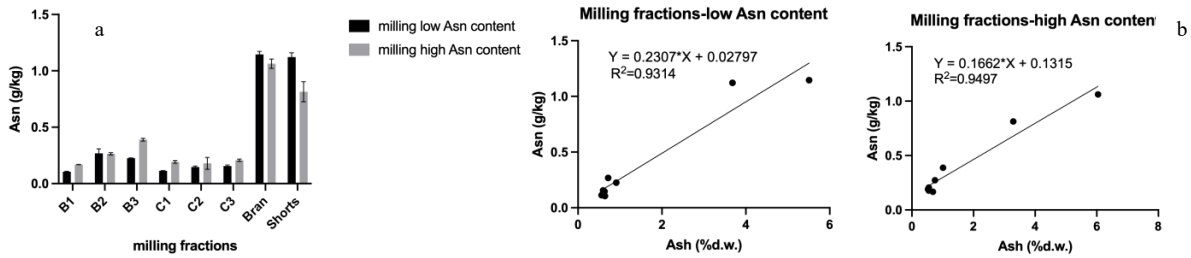

Figure 2. a*) Milling fraction obtained from milling of group with low and high Asn content (g/kg d.w.); b) Correlation between Asn and ash (%d.w.) of the milling fractions obtained from varieties with low and high Asn content.*

Results showed that it is possible to obtain flour with lower content of acrylamide precursor acting on agronomic conditions; moreover, through appropriate recombination of different milling flour, it is possible to realize flours with low Asn content even starting from a kernel with high Asn content.

4.2 Evaluation of different processing conditions on food contaminants formation in Neapolitan pizza

Flours obtained as reported in paragraph 4.1 were used as a raw material to produce Neapolitan pizza; evaluation of acrylamide and PAHs content of pizza samples has been carried out to assess the effect of wood fired oven and electric oven on food contaminants' formation. To date, no data concerning PAHs content of Neapolitan pizza are available in literature to the best of the author's knowledge, and therefore, it was investigated and prioritized in this contribute (data concerning acrylamide will be discussed during the oral presentation).
Flour "0", "00" types, whole wheat flour type and the derived doughs were analyzed to test for the background PAHs levels of raw materials used for pizza production. Results revealed that all kind of flours and doughs analyzed were free from heavy PAHs. The most representative PAHs detected were light PAHs such as Ac, F and Pa, representing the 89, 85 and 92% of total PAHs in "00", "0" and whole wheat flour types, and 80, 78 and 78% of total PAHs in doughs obtained from these flours, respectively. The ∑ 14 PAHs was 3.4, 5 and 8.4 µg/kg d.w. in "00", "0", and whole wheat flour, respectively, and reached values of 9.7, 8 and 22,7 µg/kg d.w. in the doughs. These values were in agreement with the study of Ciecierska (2013) carried out on bread and different raw materials used for its baking. Figure 3 reported data concerning light PAHs content of pizza baked in wood fired oven and in electric oven. Considering a slice of pizza (as pizza it is usually consumed), the samples obtained with the whole wheat flour showed a significantly higher content of light PAHs ($p<0,05$), in agreement with other studies which also revealed the higher PAHs content of bran compared with other products of grain grinding (Ciecierska et al., 2013). It's interesting to note that, for the most of light PAHs analyzed, samples with topping of tomato sauce showed higher values of PAHs compared with samples without topping. The results of pizza baked by electric oven showed no significant differences in PAHs levels in comparison with pizza baked in wood fired ovens. Also in this case, heavy PAHs were under LOD for most of the analyzed samples.

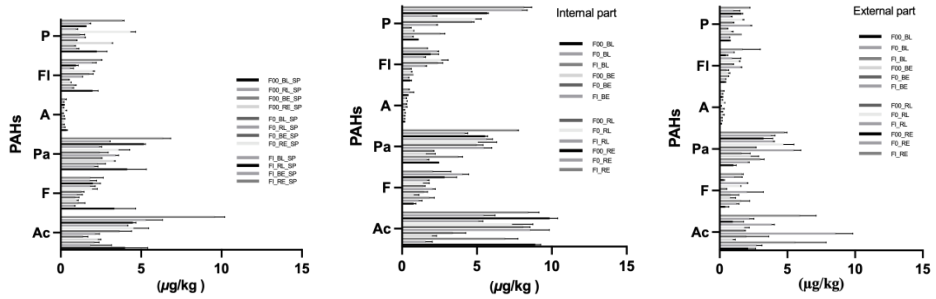

Figure 3. *Content of light PAHs (µg/kg d.w.) of pizza baked in electric and wood-fired oven (Ac=acenaphthene; F= fluorene; Pa= phenanthrene; A=anthracene; Fl=fluoranthene; P=pyrene; F00=type "00" flour; F0= type "0" flour; FI=whole bread flour; SP= slice of pizza; R=pizza with topping of tomato sauce; B=pizza without topping; L=pizza baked in wood fired oven; E= pizza baked in electric oven).*

5. Conclusion and future perspectives

The high temperature reached during baking in oven can result in the formation of food processing contaminants in pizza. The obtained results showed that it is possible to contain acrylamide formation during baking by acting on the raw materials used for pizza production. The thermal treatment and raw materials, primarily flour, are responsible for the formation of light PAHs in pizza. The Commission Regulation (UE) No. 835/201 prescribed the limit of 1 µg/kg for 4 heavy PAHs (BaP, BaA, BbF, Ch) in processed cereal-based foods, not found in the investigated product.

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Individual Variation in Food Perception and Implication in Consumer Preference of Sustainable Products

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This PhD thesis focused on the determinants of liking of novel plant-based food formulations added with innovative ingredients, exploring individual variation in consumer perception and preference to foster the transition to a healthy and sustainable diet. Sensory data were combined with technological and instrumental ones to develop innovative improved foods. In addition, a cross-national study was performed to better understand possible cross-national differences in consumer acceptance of new food formulations added with micro- and macroalgae.

Variazione individuale nella percezione del cibo e implicazioni sulla preferenza del consumatore per prodotti sostenibili

Questa tesi di dottorato si è focalizzata sulle determinanti del gradimento di formulazioni alimentari a base vegetale arricchite con ingredienti innovativi, esplorando la variazione individuale nella percezione e preferenza del consumatore per favorire la transizione verso una dieta sana e sostenibile. I dati sensoriali sono stati associati a dati tecnologici e strumentali per sviluppare alimenti innovativi migliorati dal punto di vista nutrizionale e sensoriale. Inoltre, è stato previsto uno studio cross-nazionale per meglio comprendere le possibili differenze di gradimento del consumatore guidate dal paese di origine per nuove formulazioni alimentari con aggiunta di micro- e macroalghe.

Key words: Sensory science, Consumer science, pulses, pseudocereals, algae.

1. Introduction

Current food systems are no longer sustainable. By contributing to climate change, freshwater use and biodiversity loss, food production is a major driver of global environmental change (Willet et al. 2019). Therefore, ensuring healthy food systems requires sustainable transformation of the entire food chain. In this context, the exploitation and use of minor crops, the replacement of animal proteins with those of vegetable origin, as well as the use of innovative ingredients are important strategies to increasing environmental sustainability. Among minor crops, Tartary buckwheat (*Fagopyrumtataricum Gaertn*.) is a pseudocereal that has environmental (Giupponi et al. 2019) and nutritional benefits (Ahmed et al 2014) but reduced appreciation for its bitter and astringent properties which can limit its consumption (Suzuki et al. 2004). On the other hand, pulses represent a particularly promising alternative protein source with excellent nutritional properties and require fewer natural resources than traditional crops (Nemecek et al. 2008; Preissel et al. 2015). Finally, the introduction of ingredients not belonging to the culinary culture of Western countries could contribute to the diversification in the development of new healthy and sustainable products. In this context, micro and macro algae have received particular attention in recent years, both for their ability to grow in extreme conditions (Caporgno & Mathys, 2018) and for their important nutritional profile (Khan et al., 2018; Wells et al., 2017). However, algae have some sensory characteristics (green-blue color and fishy taste and smell) that are disliked by consumers. This PhD project contributed to the sensory optimization of sustainable plant based products with high nutritional value through the study of consumers liking and expectations in order to find drivers of acceptance and/or rejection. The present PhD project consists of three main activities, two of which (A1 and A2) are part of the MIND FoodS Hub project (Lombardy Region):

A1) Study of Tartary buckwheat exploitation as an ingredient in food formulations to verify whether it is associated with the perception of unpleasant sensory characteristics (e.g. bitter and astringency) and explore its drivers of liking and rejection.

A.2) Study of the effect of health and environmental information on liking and expectations of consumers with different level of food neophobia for food formulations added with pulses (red lentils and chickpeas).

A.3) Study of cross-national differences in the acceptability as well as drivers of liking and rejection of crackers added with different micro- and macro-algae in children and adults (activity in progress).

2. Materials and Methods

A.1) Six gluten-free samples of a corn-based formulation were produced, added with 20%, 30% or 40% of either

common buckwheat (CB) or Tartary buckwheat (TB) flour (Raetia Biodiversità Alpine, Teglio, Sondrio, Italy). Sensory profiling data (8 trained assessors) were related to various instrumental analyses: electronic tongue, colorimeter CIELAB and Texture Analyzer. Subsequently, the overall liking and appropriateness of the sensory characteristics were assessed to explore drivers of liking and rejection involving 120 consumers.
A.2) Whole-corn flour (Molino Filippini S.r.l., Teglio, Sondrio, Italy) was used to prepare one control sample (100% whole corn) and two experimental samples replaced with 20% pulse flour (either chickpea or red lentil). One hundred- twenty seven consumers assessed samples overall liking in blind (tasting without information), expected (only information without tasting) and informed conditions (tasting with information) to study the effect of information on consumer expectations. To explore the effect of information on consumer clusters, questionnaires on food neophobia (fear to try new foods) and sustainable behaviour were provided.
A.3) A control sample (100% wheat) and five experimental samples with the addition of 5 % (w/w) of *Arthrospira platensis* (Green Spirulina, entire bacterium), *Arthrospira platensis* (Blue Spirulina, fractionated protein), *Palmaria palmata*, *Saccharina latissima*, or *Lithothamnium calcareum* were developed. The overall liking of the six samples, their sensory description using Check-All-That-Apply (CATA) questionnaire as well as the identification of the preferred sample were envisaged with 321 adult consumers to determine the drivers of liking and rejection and to establish which micro or macro algae were most suitable to enrich the cracker samples.

3. Results and Discussion

A.1) Sensory profiling data, partially supported by instrumental analyses (Fig. 1), revealed a higher intensity of bitterness, astringency and a darker colour in samples with high percentages of Tartary buckwheat (TB30; TB40). These properties may justify overall liking data (Fig.2) showing that samples, although well accepted, differed significantly in terms of acceptance ($F_{5,694}$=6.40, p<0.0001), with TB40 being the only one to receive lower hedonic ratings. In particular, high intensity of colour, overall flavour, bitterness and texture were identified as drivers of disliking according to PLSR analysis (Fig.3). These findings confirm that polyphenols present in buckwheat may impart astringency and bitter taste (Soares et al., 2013) as well as influence colour (Khoo et al., 2017). However, results showed that by keeping Tartary buckwheat concentration <40%, new sustainable and accepted products can be developed. Moreover, cluster analyses conducted on overall liking (results not shown), identified a non-negligible percentage of consumers (30%) who prefer the samples with the highest Tartary buckwheat additions, suggesting that there is a possible target of consumers willing to accept these products.

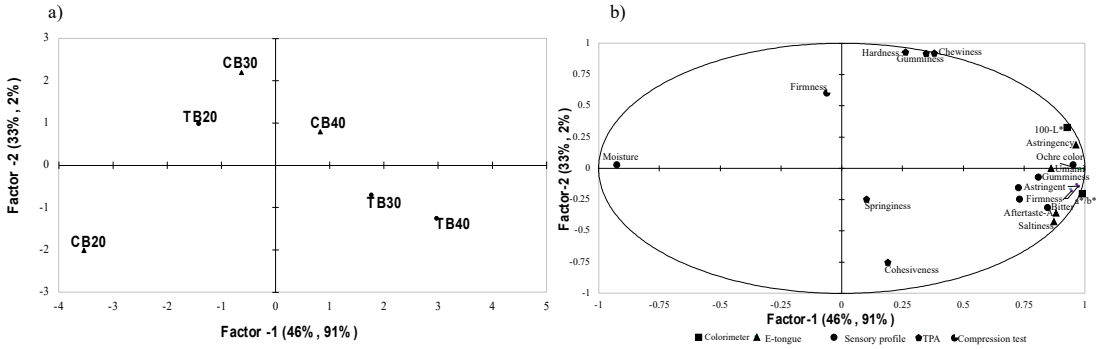

Figure 1. *Score plot (a) and loading plot (b) obtained by the PLSR model of sensory analysis and instrumental determinations for each sample (CB20, CB30, CB40, TB20, TB30, TB40) (CB = common buckwheat; TB = Tartary buckwheat). Aftertaste-A = aftertaste-astringency.*

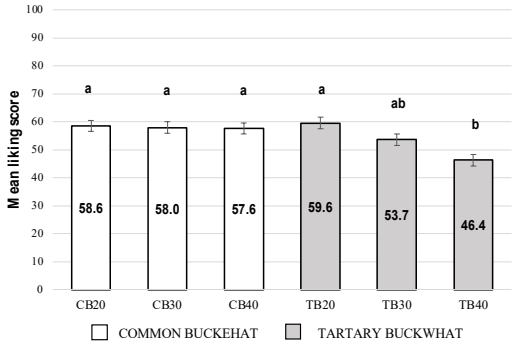

Figure 2. *Mean liking scores for Common Buckwhaet (CB) and Tartary Buckwheat (TB) samples.Different letters indicate significant differences (p< 0.0001).*

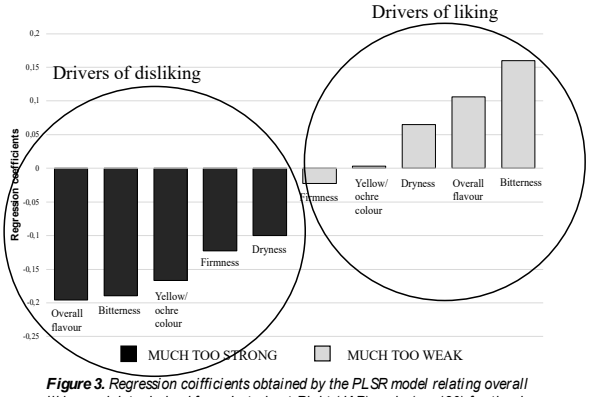

Figure 3. *Regression coiffficients obtained by the PLSR model relating overall liking and data derived from Just-about-Right (JAR) scale (n= 120) for the six samples.*

A.2) One-way ANOVA showed that samples within each experimental condition were comparable (Tab. 1) indicating no effect of type of flour on liking; overall, the samples were well accepted, with mean liking scores above the middle of the scale setted at 50. Moreover, for all samples, there was a disconfirmation of expectation (E−B >0), this means that consumers rated the products worse than their expectations (Tab. 1). This disconfirmation was associated with an assimilation effect (R−B >0) for both samples containing pulses. This result suggests that the conveyed health and environmental information influenced liking, leading consumers to change their hedonic score in the direction of their expectations. However, this assimilation was incomplete (R−E <0). These results are consistent with previous research showing that information about health (Saba et al., 2010) and sustainability (Laureati et al., 2013) may lead to increased consumer expectations and liking but sensory properties play a major role.

Table 1. *Mean hedonic scores under the 3 experimental conditions (B = blind; E = Expected; R = Real) and expectation effect on samples liking (n.s., not significant; *, p<0.05; **, p<0.01; ***, p<0.001) Disconf. = negative disconfirmation, Ass. = assimilation. (1) Comparison by column (samples within B,E or R condition) are based on 1-way ANOVA; (2) Comparison by row (conditions within samples) are based on paired t-test.*

Samples	Scores			E−B (2)		R−B (2)		R−E (2)	
	B	E	R	Mean	p-value	Mean	p-value	Mean	p-value
CONTROL	62.7	76.2	65.0	13.5	*** (Disconf.)	2.3	n.s.		
20% CHICKPEAS	60.2	73.2	67.3	13.0	*** (Disconf.)	7.1	** (Assim.)	−5.9	* (Incomplete)
20% RED LENTILS	58.9	72.2	65.4	13.3	*** (Disconf.)	6.5	** (Assim.)	−6.8	** (Incomplete)
	n.s. (1)	n.s. (1)	n.s. (1)						

The same information affected differently consumers hedonic responses according to their food neophobia index (Tab. 2). A negative disconfirmation of expectation (E−B >0) was found only for Neophilic and Neutral groups. This disconfirmation was associated with a complete assimilation effect for the red lentil sample for Neophilic people and for chickpea sample for Neutral ones, while incomplete assimilation was found for red lentil sample for Neutral subjects. No significant differences between blind and expected conditions were found for the Neophobic group (Tab. 2). This result can be attributed to the fact that neophobic people are individuals with low reactivity towards novel food products (Tuorila and Hartmann, 2020) and more generally, have little interest in food (Jaeger et al., 2017), even when such food is accompanied by positive information.

Table 2. *Mean hedonic scores provided by Neophilic, Neutral and Neophobic consumers scores under the 3 experimental conditions (B = blind; E = Expected; R = Real) and expectation effect on samples liking (n.s., not significant; *, p<0.05; **, p<0.01; ***, p<0.001) Disconf. = negative disconfirmation, Ass. = assimilation.*

FNS groups	Samples	Ratings			E−B		R − B		R − E	
		B	E	R	Mean	p-value	Mean	p-value	Mean	p-value
Neophilic	CONTROL	63.2	78.0	66.2	14.8	** (Disconf.)	3.0	n.s.		
	20% CHICKPEAS	64.6	79.8	71.1	15.2	*** (Disconf.)	6.5	n.s.		
	20% RED LENTILS	60.5	78.7	70.3	18.2	*** (Disconf.)	9.8	* (Assim.)	−8.4	n.s. (Complete)
Neutral	CONTROL	60.3	76.9	64.6	16.6	*** (Disconf.)	4.3	n.s.		
	20% CHICKPEAS	58.7	72.0	66.7	13.3	*** (Disconf.)	8.0	* (Assim.)	−5.3	n.s. (Complete)
	20% RED LENTILS	58.6	72.5	65.2	13.9	*** (Disconf.)	6.6	* (Assim.)	−7.3	* (Incomplete)
Neophobic	CONTROL	67.8	72.7	64.8	4.9	n.s.	−3.0	n.s.		
	20% CHICKPEAS	59.7	69.3	65.0	9.6	n.s.	5.3	n.s.		
	20% RED LENTILS	57.9	65.2	61.0	7.3	n.s.	3.1	n.s.		

A.3) Two-way ANOVA revealed that respondents across all countries provided on average positive liking scores towards all the algae enriched crackers, with Italian respondents providing significantly higher liking scores compared to Belgium and Sweden (F_{Sample} =24.2; p<0.0001; $F_{Country}$ = 14.8; p<0.0001; $F_{Sample*Country}$ =7.7; p= p<0.0001) (Fig. 4). These results are supported by previous work in the literature that has established that food preferences differ between countries (Baharuddin et al., 2015). Moreover, Principal Coordinate Analysis revealed that, for all countries, characteristics such as "toasted bread flavor", "salty" and "crunchy" had positive impact on liking. For Belgium and Sweden rejection drivers were related to "off-flavor", while for Italy to "hard texture" and "speckled appearance". For Italy, "green color" was a driver of acceptance (Fig. 5). The same products are currently being tested with children from different EU countries.

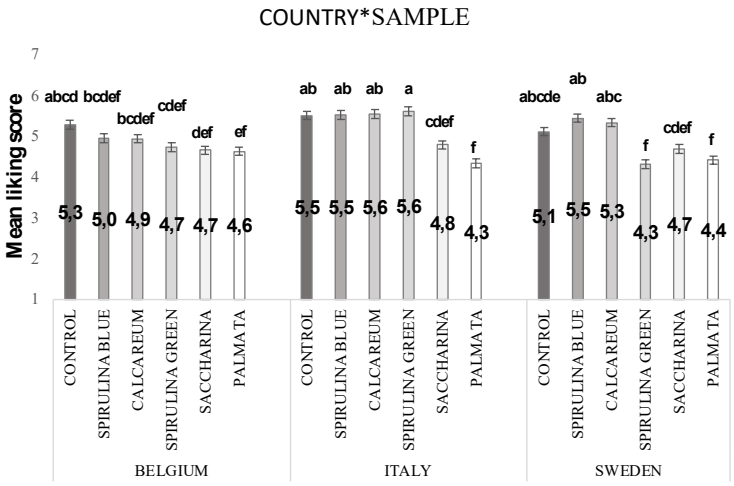

Figure 4. *Mean liking scores for the control samples and the crackers enriched with algae in each country. Different letters indicate significant differences (p< 0.0001).*

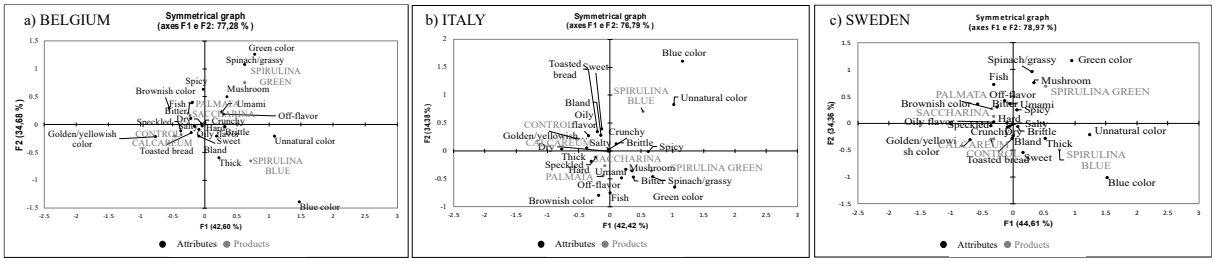

Figure 5. *Symmetric plot derives from Principal Coordinate Analysis (PCA) based on CATA description of 6 crackers for the whole sample of a) Belgian, b) Italian and c) Swedish consumers (n=321).*

4. Conclusions and Future Perspectives

This PhD project, through 3 main studies, has contributed to a better understanding of the factors involved in consumer acceptability of new food formulations added with sustainable and innovative ingredients. The outcome of this PhD project represent an effective contribution to food companies interested in developing new healthy and sustainable products. The results obtained show that: A.1) Tartary buckwheat can be added to corn-based gluten-freeformulations in concentrations up to 30% without compromising consumer acceptance. However, it is suggested to add this pseudocereal also to more widely consumed products than the one used in our study. A.2) Associating new sustainable ingredients with beneficial health and environmental information can be a strategy to improve consumer liking and expectation potentially contributing to promote their consumption. However, health and environmental information is completely ineffective for individuals with a higher food neophobia level. This indicates the need to consider consumers' individual characteristics and to develop appropriate and personalized communication strategies when specific innovations are launched on the market. A.3) The addition of micro- and macro-algae in crackers seems promising, however, country of origin has a significant effect on liking. Data on children from the same European countries is being collected to investigate the acceptability as well as drivers of liking and rejection of crackers added with micro- and macro-algae in another population's segment.

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Novel Insights on the Functional and Nutritional Features of the Foods Based on Cereals and Legumes

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Tutor: Prof.ssa Calasso Maria

This research project aims to improve foods' nutritional and functional characteristics based on cereals and legumes, using biotechnological strategies to exploit unconventional matrices. First, type I sourdough obtained from sprouted wheat grains and lentils were studied. The second activity concerned the study of gluten-free bread (GF) obtained using type II sourdough and enriched in an artichoke by-product. Finally, self-structuring drinks obtained from the combination of plant protein isolates (soy, pea, lentil) and hydrocolloids (hydroxypropyl-methylcellulose, xanthan, guar gum, sodium alginate, pectin) were studied.

Nuovi approfondimenti sulle caratteristiche funzionali e nutrizionali degli alimenti a base di cereali e legumi

Questo progetto di ricerca ha come obiettivo uno studio sul miglioramento delle caratteristiche nutrizionali e funzionali di alimenti a base di cereali e legumi, utilizzando strategie biotecnologiche per sfruttare matrici non convenzionali. Inizialmente, sono stati studiati lieviti naturali di tipo I ottenuti da granelle germinate di frumento e lenticchia. La seconda attività ha riguardato lo studio di pani senza glutine (GF) ottenuti utilizzando lievito naturale di tipo II e arricchiti con sottoprodotto della lavorazione del carciofo. Infine, sono state studiate bevande auto-strutturanti ottenute combinando isolati proteici vegetali (soia, pisello, lenticchia) con idrocolloidi (idrossipropilmetilcellulosa, xantano, gomma guar, sodio alginato, pectina).

Key words: sourdough; germination; legumes; artichoke; plant-protein isolate

1. Introduction

Cereals, legumes and related foods are an important source of energy, as well as a range of non-nutrient bioactive components that provide health benefits. The main challenges for the near future include the exploration of non-conventional matrices and the implementation of processing and biotechnological strategies (such as germination and fermentation) finalized to improve their functional and nutritional properties. In accordance with the PhD thesis project, this communication reports the main results of three activities concerning:

(A1) Evaluation of physicochemical, microbiological, metagenomics, metatranscriptomics and metabolomics parameters during the preparation and propagation of firm and liquid sourdoughs obtained by traditional fermentation (type I sourdough) and backslopped over 10 days with sprouted and non-sprouted flours in order to assess its potential use in bread making;
(A2) Nutritional and functional evaluation of gluten-free bread obtained by using type II sourdough and enriched in an artichoke by-product;
(A3) Application of hydrocolloid technology for developing self-structuring beverages plant-protein based.

2. Materials and Methods

2.1 Characterization of firm and liquid sourdough from sprouted and non-sprouted grains

Two different grains were considered in this study: a first made of a cereal (wheat, *Triticum durum* var. Simeto), and a second composed of a legume (lentil, *Lens culinaris*). Cereals and legumes were purchased from local markets. The wheat and lentil sprouting processes were performed according to Montemurro *et al.,* (2019) with some modifications described by Perri *et al.,* (2021). Sprouted wheat and lentil grains were milled into smaller particle sizes (< 500 μm) by using a laboratory mill (Ika-Werke M20 GMBH, and Co. KG, Staufen, Germania), without removing the rootlets thus obtaining the whole sprouted wheat and whole sprouted lentil flours using to obtain sprouted wheat and sprouted lentil sourdough (SW, SL). The non-sprouted wheat flour was obtained from whole non-sprouted wheat grains through the same laboratory mill in order to obtain non-sprouted wheat sourdough (NSW). In addition, sourdough obtained from commercial refined wheat flour (RW) was used. The preparation of dough and propagation of sourdough was performed by a traditional protocol without the addition of starter cultures or baker's yeast. Spontaneous wheat and lentil sourdough fermentations were carried out through backslopping, both in firm and liquid conditions (dough yield 160 and 280 respectively). Based on previous work (Rizzello *et al.,* 2014), a flour composition has been selected to produce sprouted wheat-lentil

sourdough (SWSL). Culture-dependent microbiological characterization by using plate count and the study of community-level physiological profile (CLPP) by using OmniLog MicroStation was studied in the dough before fermentation (D0) after the first fermentation of 24h (D24) and after 3, 6, and 10 days of refreshments. Biochemical (pH, total titratable acidity (TTA), lactic acid, acetic acid, fermentation quotient) and nutritional characterization (total phenolic concentration, antioxidant activity and antinutritional factors (phytic acid and raffinose)) of dough and sourdoughs at D0, D24 and R10, were carried out. For metagenomic analysis, 16S rRNA gene amplicons of the bacterial communities of doughs and sourdoughs using primers 350F/814R, targeting the region V1-V3 of Firmicutes were sequenced by Illumina 2×300bp paired-end MiSeq platform. 16S sequencing-derived fastQ files were checked for quality using FastQC software. In silico bioinformatics analyses, including denoizing, taxa assignment and alpha and beta diversity, relied on the QIIME2 (Bolyen *et al.,* 2019) microbiome platform (version 2020.8). Quantification of total bacteria and specific species was performed on the D0, D24 and R10 collected samples, by qPCR following the method reported in Pontonio *et al.,* (2017) and Kwok *et al.,* (2014) with some modifications. qPCR was performed on a 7300 Real-Time PCR System (Applied Biosystem, Forter City, CA USA). Metatranscriptomics analysis was carried out on all mature sourdoughs (R10) by using Illumina NextSeq 500 sequencing platform (Illumina, San Diego, CA, USA). The raw metatranscriptomic sequencing data (reads) of all sourdough at R10 were analysed in silico using SqueezeMeta pipeline Version 1.0, July 2019 together with other ad hoc utilities developed to manage assembly and annotation in order to obtain taxonomic information and metabolic pathways involved. Metabolomics profile was also detected and data analysis is still ongoing. Based on metadata sample stratification a two-group Welch corrected test was used for group pair comparisons and only statistically significant results were kept. All performed analyses were corrected for multiple tests by applying the Benjamini-Hochberg procedure. Error bar plots have been obtained by using the STAMP software (Parks *et al.,* 2014).

2.2 Nutritional and functional evaluation of gluten-free bread enriched in an artichoke by-product

Type II sourdough (tIISD) dough yield 200 (DY200) was obtained by using commercial rice flour. *Leuconostoc pseudomesenteroides* (DSM20193) was inoculated at cell density approx. 10^7 CFU/g. Four different gluten-free bread (DY200) were prepared. In detail: i) gluten-free leavened bread without the addition of sourdough and artichoke extract (YB), ii) gluten-free leavened bread with the addition of artichoke extract (YB-AE), iii) gluten-free bread leavened with tII-SD (SB), iv) gluten-free bread with tII-SD and addition of artichoke extract (SB-AE). All the loaves were baked at 210°C for 30min. The loaves were subjected to *in vitro* digestion to evaluate the predictive glycemic index (PGI), according to Liljeberg *et al.,* (1996). For the evaluation of the antioxidant activity DPPH (2,2-diphenyl-1-picrylidrazyl), methanol extracts (ME) were first obtained from each sample, according to Perri *et al.,* (2021). Then, ABTS [2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] test was conducted as a control. Microbiota and volatile profiling of fermented faecal batches were carried out. To perform the *ex vivo* experiments, two different human cell lines were used, both provided by the National Institute for Cancer Research of Genoa (Italy). Specifically, the cell lines were Caco-2 (colon adenocarcinoma) ICLC HTL97023 and the human keratinocytes NCTC 2544. The viability of cells tested for toxicity has been assessed. In addition, the pro-inflammatory contribution of bread digests was evaluated by estimating levels of TNF-α and interleukin 1-β expressed in Caco-2 cells.

2.3 Application of hydrocolloid technology for developing self-structuring beverages plant-protein based

The following activity is part of a period of study and research abroad (still ongoing) at the Department of "Nutritional and Food Science" of University College Cork. The activity involves the development of innovative laboratory-scale satiety-enhanced beverages with high protein/fibre content.

3. Results and Discussion

3.1 Ecology dynamics in spontaneous sourdoughs made from native and sprouted wheat and lentil flour

With the aim of ascertaining the impact of the sprouting process and the dough yield condition on sourdoughs, all the obtained replicates corresponding to the starting point (D0), the first day of backslopping at 30°C (D24) and the 10th day of refreshment at 30°C (R10), have been inspected in terms of culture-dependent, biochemical, nutritional, metataxonomic metatranscriptomics and metabolomics profile. Culture-dependent approaches are shown in Fig. 1A. The cell density of the main microbial groups was affected by matrix, time of collection and condition of propagation. LAB cell densities at D0 were 2 or 3 log cycles highest in doughs made by flours obtained from sprouted grains. The yeast's initial cell density increased during propagation and reached 5.0 ± 0.29 log CFU/g at the end of the 10th daily refreshment. Sprouted sourdoughs harboured a higher number of yeasts. A similar trend was previously observed in the spontaneous fermentation of sprouted lentil flour alone or in a mix with cereal flour (Perri *et al.,* 2021). In line with Ercolini and co-workers (Ercolini, 2013), presumptive Enterobacteriaceae were enumerated in each dough which results higher in sprouted samples ($p < 0.05$) and were no longer detected on day 10 of sourdough propagation. When CLPP profiles were checked, the sprouted matrices revealed an increase in the metabolisms related to carbohydrates at the 10th refreshment time point. Furthermore, when compared with RW, the SWSL matrix showed a higher amino acid metabolism profile. Prior

to the fermentation, higher pH and lower TTA values in liquid samples were observed when compared with firm ones. Moreover, despite not being supported by statistical significance, we noticed a decreasing pH trend for liquid sourdoughs till the end of the fermentation process with pH values lower than 4.5. These results agree with previous evidence (Di Cagno *et al.,* 2014), stating how liquid sourdoughs obtained only from raw wheat showed lower pH values at the end of the fermentation. By inspecting the sprouted group, sprouted samples showed higher pH values at the 10th refreshment. Moreover, when SW and SWSL were both compared against RW, a lower amount of organic acids (acetic and lactic) marked sprouted samples, as evidenced by the lower fermentation quotient measured at the end of the fermentations. The total phenolic concentration and the antioxidant activity reported a not significant increase ($p > 0.05$) at the end of the 10 daily propagation. A lower amount in sprouted samples of raffinose, a fermentable sugar included in the class of fermentable oligosaccharides, disaccharides, monosaccharides and polyol (FODMAP), would prevent gastrointestinal symptoms (Curiel *et al.,* 2015). At the same time, phytic acid which chelates divalent minerals and reduces their bioavailability, was detected at lower amounts in sprouted and mature sourdough (Curiel *et al.,* 2015). Although we found only a lowering trend describing the phytic acid decrease at the end of fermentation, our data revealed a statistically significant lower value of raffinose in sprouted than non-sprouted samples. It is known that different strains of lactic acid bacteria (LAB) belonging to the Lactobacillus genus, can produce in sourdough the α-Galactosidase enzyme useful to reduce the raffinose family oligosaccharides (RFO's) content in different products (Yoon & Hwang, 2008). qPCR results detected several species of lactobacilli whose presence could justify raffinose level reduction in mature sourdoughs (R10). As evidenced by alpha and beta diversity estimates, we noticed how both the Welch and PERMANOVA relative statistics proved a different distribution of taxa based on the matrix stratification. A higher relative abundance of *Pediococcus* genus was found in the SWSL when metagenomic data were inspected. NSW was found to be significantly enriched in *Empedobacter*. A significantly increased relative abundance of *Weissella* in NSW was also found. A significant abundance of *Enterococcus faecalis* emerged from the comparison of both SW and SWSL against RW. The results on sourdough are in line with our previous evidence on SW-germinated flour Perri *et al.,* (2020). *Enterococcus faecalis* exerts an important role in the production of γ-aminobutyric acid (GABA) (Khanlari *et al.,* 2021), an aspect that deserves further investigation. Regarding metatranscriptomics, starting from the raw sequencing data (raw reads), it is possible to perform assembly and annotations in the file "ORF table" (SQM project) that will allow the creation of database taxa/ specific functions. The inspection of the annotation data through different filters has been fundamental for the recovery of unique/shared functions up to the taxonomic level of the species and to different levels of metabolic pathways of the KEGG. Fig. 1B shows the comparison between NSW (non-sprouted wheat) and SW (sprouted wheat) matrices. It emerged that oxidative phosphorylation metabolism is exclusive to the sprouted matrix. The ABC transporters metabolism has a greater occurrence of KEGG while less occurrence of KEGG characterized the lipoic acid metabolism and in the phenylalanine/tyrosine/tryptophan biosynthesis metabolism. The microbial profile associated with each metabolism appears significantly different: *Comamonas terrigena*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Latilactobacillus curvatus*, *Latilactobacillus sakei*, *Leuconostoc citreum*, *Levilactobacillus brevis*, *Pediococcus pentosaceus*, *Pseudomonas putida*, *Weissella cibaria*. The ABC complex transporters metabolism showed a high KEGG occurrence associated with *Lactiplantibacillus plantarum* while *Pediococcus pentosaceus* do not present KEGG occurrence.

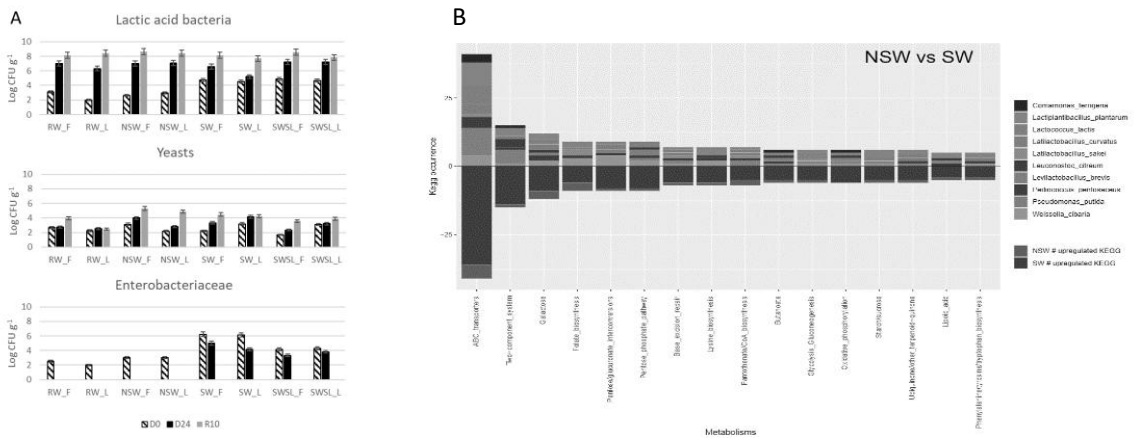

Figure 1 *A) cultivable bacteria and yeast cell density (log CFU g^{-1}) for each analysed sample. RW, NSW, SW, and SWSL density in firm and liquid conditions (F, L) at three collection times (D0, D24 and R10): microbial growth trends for the group of presumed lactic acid bacteria, yeasts and Enterobacteriaceae. B) Bar plot obtained from comparing the ORF table data between two matrices (NSW non-sprouted wheat and SW sprouted wheat). The graph shows each species' significant occurrence values of KEGG (Y-axis) for a given metabolism (X-axis).*

Based on high-resolution metabolomics and metatranscriptomics merged results we were confident in detecting the contribution of statistically significant altered transcript at the species level. Metabolic sub-pathways belonging to carbon (starch/sucrose/galactose metabolism, pentose phosphate pathway, pentose glucuronate interconversion) and nitrogen (arginine/lysine/glycine) metabolisms, as well as, cell-cell communication (two-component system and quorum sensing), were checked also in terms of metabolites by reconstructing the step-by-step reaction flow.

3.2 Nutritional and functional evaluation of gluten-free bread enriched in an artichoke by-product

This study aimed to modify a traditional food consumed daily to support patients with gluten-related disorders (GrD) and those with proven celiac disease (CeD). The predicted glycaemic index (PGI) was calculated on digested bread samples. Values between 62.8-111.3 (Fig. 2A) were found in SB and YBAE, respectively. A decrease in PGI was found in samples containing tII-SD ($p < 0.004$) since microbial fermentations are well-known as a good way to metabolize sugar-containing substrates (De Vuyst *et al.,* 2021). The presence of AE significantly increases PGI in SB-AE. By setting a water-based extraction protocol, Garcia-Castello *et al.,* (2022) enlightened how it was possible to recover 60% of the polyphenolic quantity and 56% antioxidant activity from the solid waste of artichoke by-products. As shown in Fig. 2B, the paired comparison of bread with AE against bread without AE shows DPPH values 15 times higher in AE bread ($p <0.001$). A comparison of SB-AE and YB-AE shows that the first bread has higher antioxidant activity ($p = 0.02$). ABTS test confirms the DPPH test. Fig. 2C shows the principal component analysis (PCA) results concerning the microbiota analysis of the faecal batches fermented at 20 and 42h. Based on the PC1 results, samples at T20 and T42 are plotted in the positive and negative quadrants, respectively, indicating a partial grouping in time. At T20, samples show increased viability of total bacteria (aerobic and anaerobic TBC), *Enterobacteriaceae* and LAB. At T42, it increases the viability of *Streptococcus*, *Clostridium* and *Bifidobacterium*. Few differences were found in AE-containing bread. At T42, *Bifidobacterium* and *Clostridium* are abundant in YB, and adding AE does not change the trend. Instead, SB mainly hosts LAB and streptococci without significant differences with the addition of AE. The metabolic profile of the faecal batches after 20 and 42h of incubation was different in qualitative and quantitative terms. 59 volatile compounds have been identified in the following classes of chemical compounds: alcohols (5), aldehydes (10), esters (6), hydrocarbons (6), indoles (2), ketones (8), organic acids (14), phenols (2) and terpenes (2). In addition, 4 compounds not belonging to the above classes have been identified: 3-Ethyl-3-methylheptane; 1H-pyrrole-2,5-Dione; 3-ethyl-4-methyl, 8-methyl nonanoic acid; γ-Dodecalactone. Specifically, after 20 hours of faecal fermentation, we distinguish SB from YB by the presence of the latter of aldehydes and hydrocarbons, without significant differences resulting from the addition of AE. High levels of hydrocinnamic acid were detected in SB-AE-T20, one of artichoke's most representative phenolic compounds (Pandino *et al.,* 2012). The VOCs profile changed partially after another 22 hours of incubation. In fact, at 42h a wide spectrum of organic acids, phenols and indoles has characterized samples containing AE differently. Based on *ex vivo* experiments, SB and AE combined decreased the cellular TNF-α and IL1-β expression.

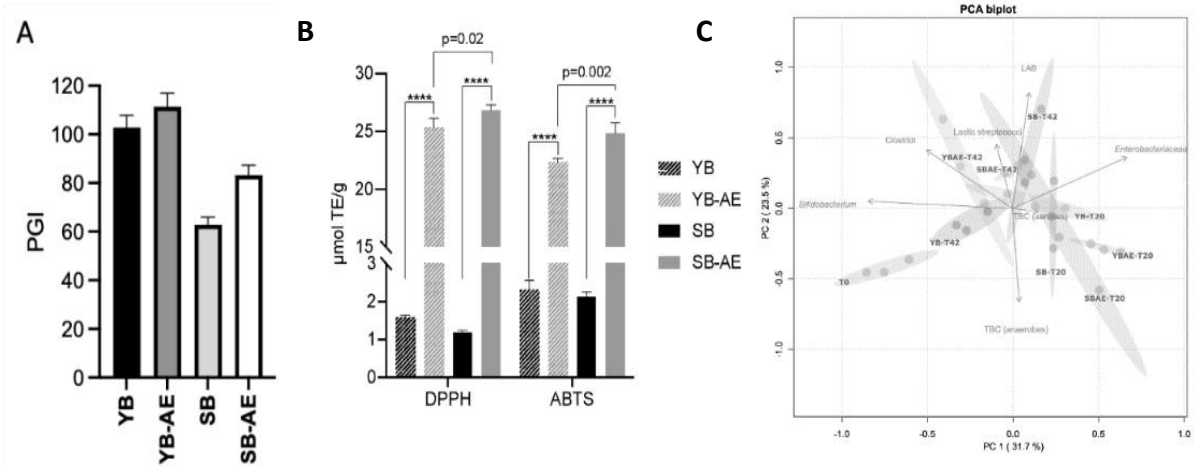

Figure 2 *A) predicted glycemic index (PGI) of digested breads made with or without type-II sourdough (S and Y, respectively) and with or without a powdered artichoke extract (AE); B) radical scavenging activity, based on DPPH and ABTS assays, of breads. Results were expressed as µmol of Trolox equivalent (TE)/g. "****" means p-value < 0.001; C) Biplot of principal component analysis (PCA) on the microbial density of faecal batches fermented with four different loaves (YB; YB-AE; SB; SB-AE) in two fermentation times (20 and 42 hours, T20 and T42, respectively). The cell density of the investigated microbial groups is also shown. Abbreviations: total bacterial count (TBC), lactic acid bacteria (LAB).*

3.3 Application of hydrocolloid technology for developing self-structuring beverages plant-protein based

Since we live in an obesogenic world, it is known that foods with satiety sensations furnished have obvious benefits for weight management and could improve the health of consumers. The aim of the research is to create ingredients that could provide nutritional enrichment to the finished product. Prototypes of smoothies will be developed using hydrocolloids and plant protein isolate which can develop with self-structure within the gastrointestinal tract providing a greater sense of gastric stuffing. It will be necessary to characterize the stability of the beverage formulation in response to different environments (pH, salt conditions, temperature). Data analysis is still ongoing.

4. Conclusions

The preliminary data shows how we could ascertain the better nutritional quality and relative shelf-life throughout the sprouted grain and mixed samples. In addition, we have seen how using by-products such as artichoke leaf extract could be a functional ingredient for developing new gluten-free products with better biological properties. This PhD project will conclude with the study results of a gluten-free smoothie prototype for its use as an ingredient with high nutritional value.

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Antimicrobial and antibiofilm activities of pomegranate phenolic compounds against foodborne pathogenic bacteria

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This Ph.D. thesis research project is aiming to investigate the antimicrobial, antibiofilm, and anti-quorum sensing capacities of the pomegranate peel extracts (PPE) on several foodborne pathogenic bacteria, together with analyzing the chemical composition of the PPE's bioactive compounds and their antioxidant activities. Bioassay-guided fractionation was used for identifying the most active natural products in the top-performing extracts. These fractions were then assessed for cytotoxicity using LDH cytotoxicity assay against human keratinocytes (HaCaTs) was performed. Finally, the effectiveness of the top-performing extracts on food matrix against *Staphylococcus aureus* was evaluated.

Le attività antimicrobiche e antibiofilm dei composti fenolici della buccia di melograno contro i batteri patogeni di origine alimentare

Questo progetto di ricerca di tesi di dottorato ha lo scopo di indagare le capacità di rilevamento antimicrobico, antibiofilm e anti-quorum degli estratti di buccia di melograno (PPE) su diversi batteri patogeni di origine alimentare, insieme all'analisi della composizione chimica dei composti bioattivi del PPE e delle loro attività antiossidanti. Il frazionamento guidato dal saggio biologico è stato utilizzato per identificare i prodotti naturali più attivi negli estratti con le migliori prestazioni. Queste frazioni sono state quindi valutate per la citotossicità utilizzando il test di citotossicità LDH contro i cheratinociti umani (HaC, aTs). Infine, è stata valutata l'efficacia degli estratti più performanti sulla matrice alimentare contro lo *Staphylococcus aureus.*

Key words: Punica granatum, agrobiodiversity, OPLS-DA, punicalagin, *Staphylococcus aureus*

1. Introduction

In accordance with the Ph.D. thesis project previously described (Amira, 2022), this oral communication reports the main results of the conducted activities concerning:
A1. Extraction of PPEs from pomegranate fruit peels.
A2. Determination of the antimicrobial activity of the PPE.
A3. Determination of antibiofilm and anti-quorum sensing.
A4. Phytochemical composition analysis of the PPE bioactive compounds.
A5. Bioassay-guided fractionation approach for isolating the most active natural products in the top-performing extracts.
A6. Cytotoxicity assessment of human cells using LDH cytotoxicity assay of treated human keratinocytes (HaCaTs).
A7. Testing the effectiveness of the top-performing extracts on food matrix against staphylococcus aureus.

Pomegranate (*Punica granatum* L.) is one of the oldest species of domesticated fruit world (Schwartz et al., 2009) which is rich in bioactive phytochemicals known for their antioxidant, anti-inflammatory, antimicrobial, and anticancer properties. Its peel which constitutes about 50% of the whole fresh fruit, contains the highest concentration of phenolic compounds, mainly hydrolyzable ellagitannins, anthocyanins, and flavonoids (Akhtar et al., 2015). As a consequence, this part of the pomegranate, considered a by-product in the agri-food sector, should instead be designated a co-product from which a host of chemical compounds can be extracted for numerous applications as food additives, nutraceuticals, and supplements in the pharmaceutical, food, and cosmetics industries (Puneeth and Chandra, 2020; Gigliobianco et al., 2022). Hydrolyzable ellagitannins are the predominant phenolic compounds in pomegranate peel, and they are also the constituent phytochemicals exhibiting the highest antioxidant capacities (Gigliobianco et al., 2022). Moreover, the magnitude of the antioxidant and antitumor activities of pomegranate peel extract are stronger than the sum of the individual activities of its constitutive bioactive molecules, indicating a possible synergistic effect resulting from the mixtures of phenolic compounds present in the pomegranate (Orgil et al., 2014; Kandylis and Kokkinomagoulos, 2020).

Infectious diseases and food decomposition caused by pathogenic microorganisms are two of the principal causes of morbidity and death worldwide (Celiksoy and Heard, 2021). Notably, food poisoning is predominantly

linked to bacterial contamination by Gram-negative bacteria and Gram-positive bacteria (Mostafa et al., 2018). The widespread use of antibiotics to control life-threatening infectious diseases in humans and animals has resulted in the rise and spread of antibiotic-resistance mechanisms among bacterial pathogens. (Slobodníková et al., 2016). Thus the search for natural antimicrobials, especially plant-derived compounds, as an alternative to artificial antimicrobial products for the treatment of certain enteric infections is presently enjoying a surge in research attention (Xu et al., 2017).

2. Materials and Methods

The chemical composition and antimicrobial activities of the peel from seven pomegranate varieties were evaluated. Dried bulk specimens were ground into a fine powder and extracted by aqueous decoction in two different levels of temperature and maceration in ethanol of the seven different pomegranate varieties. PPE-total phenolic content was assessed by Folin-Ciocalteu assay where total flavonoids were quantified by colorimetric assay according to the AlCl3 method and analysis of condensed tannins was carried out by vanillin assay. The antioxidant capacity of pomegranate extract was evaluated using both DPPH and ABTS methodologies and finally, Reverse-phase HPLC analysis of phenolic compounds was performed using an Agilent 1100 Liquid Chromatography (LC) system.

The antimicrobial activities of PPE were quantitatively evaluated in vitro by measuring the Minimum inhibitory concentrations (MICs) of the seven PPEs were estimated on different foodborne pathogenic bacteria. The antibiofilm activity was assessed using a crystal violet (CV) assay with some modifications. Bioassay-guided fractionation approach for isolating and identifying the most active natural products in the top-performing extracts, using PREP-HPLC with the goal of the identification of a single bioactive compound and Putative matches of compounds elucidation via LC–FTMS for the best-performing extracts. Cytotoxicity assessment of human cells using LDH cytotoxicity assay of treated human keratinocytes (HaCaTs) was performed. Finally, the effectiveness of the top-performing extracts on food matrix against *staphylococcus aureus* was evaluated.

3. Results and Discussion

3.1 chemical composition of PPE

The data show wide content variability between cultivars, In agreement with Whang et al. (2011), we found extraction with water at 40 °C for 4 hours to be an efficient method for the extraction of pomegranate peel antioxidants However, our data also show that extraction at this higher temperature was not always accompanied by a higher concentration of extracted compounds compared with extraction at room temperature tab 1. Eighteen phenolic compounds were identified by HPLC-DAD, and the levels of total phenols, flavonoids, and tannins are in line with the previous literature. Saad et al. (2012). Two out of the seven tested cultivars were identified as the varieties that differed from the others the most. To highlight the varietal-specific features of PPE further, models were generated investigating a single variety vs all others. Specifically, three PPEs stood out for their overall low concentration of phenols, tannins, and flavonoids. On the other hand, two PPEs were distinguished by their absence of anthocyanins and good presence of flavonoids and phenolic acids, specifically catechin, rutin, and caffeic acid, and two varieties presented high concentrations of punicalagin isomers.

The antioxidant activities of PPEs obtained at the two different extraction temperatures were generally similar. The differences in values obtained between the two extraction temperatures were even lower for DPPH values,. As for ABTS, two varieties were demonstrating the greatest differences, with hot water extraction resulting in higher phenol concentrations (Fig. 1). Previous studies have attributed the main antioxidant activity of PPE to punicalagins, punicalins, and ellagic acids (Rosas-Burgos et al., 2017). In contrast, our findings attributed a negligible role to the latter two compounds and only a secondary role to punicalagins. Instead, our results indicate overall antioxidant activity as partially related to the presence of flavonoids epicatechin, catechim, and rutin, and principally related to the levels of total flavonoids and total phenols.

3.2 antimicrobial activity of PPE

Most of the PPEs showed some effectiveness at suppressing microbial growth, in addition, the MIC values recorded in the present study (3 to 0.09 mg mL^{-1}) were much lower than those reported in previous studies (Wafa et al., 2017; Nasreddine et al., 2018).and Gram-negative bacteria notably more resistant than Gram-positive bacteria this is in agreement with results obtained from (Alexandre et al., 2019). PLS-DA analysis enabled us to identify the bioactive compounds contributing the most to the antimicrobial activities of PPE. Table 1 reports the molecules associated with the antimicrobial activity for each microbial strain.

Table 1 *List of the variables mostly related to the antimicrobial activity of the PPE according to the PLS-DA models performed per each bacterial strain tested.*

Strain	***Variables***
S. aureus 20231	Chlorogenic acid, Total Tannins, Punicalagin β, Punicalagin α
S. aureus 2569	Total Tannins, Total Phenols, Epicatechin, Total Flavonoids
S. aureus 6948	Punicalagin β, Punicalagin α, Catechin, Total Flavonoids, Chlorogenic acid
L. monocytogenes 15675	Chlorogenic acid, Total Phenols, Epicatechin, Punicalagin α, Total Tannins, Punicalagin β
L. monocytogenes 20600	Total Tannins, Total Flavonoids, Chlorogenic acid, Punicalagin β, Epicatechin, Punicalagin α
E. coli 4415	Punicalagin α, Punicalagin β, Total Tannins, Rutin, Catechin
Lac. Paracasei Shirota	Total Tannins, Epicatechin, Total Flavonoids, Total Phenols, Punicalagin α, Punicalagin β, Rutin
Lim. Reuteri 17938	Epicatechin, Total Flavonoids, DPPH, Caffeic acid, Punicalagin α, Punicalagin β,

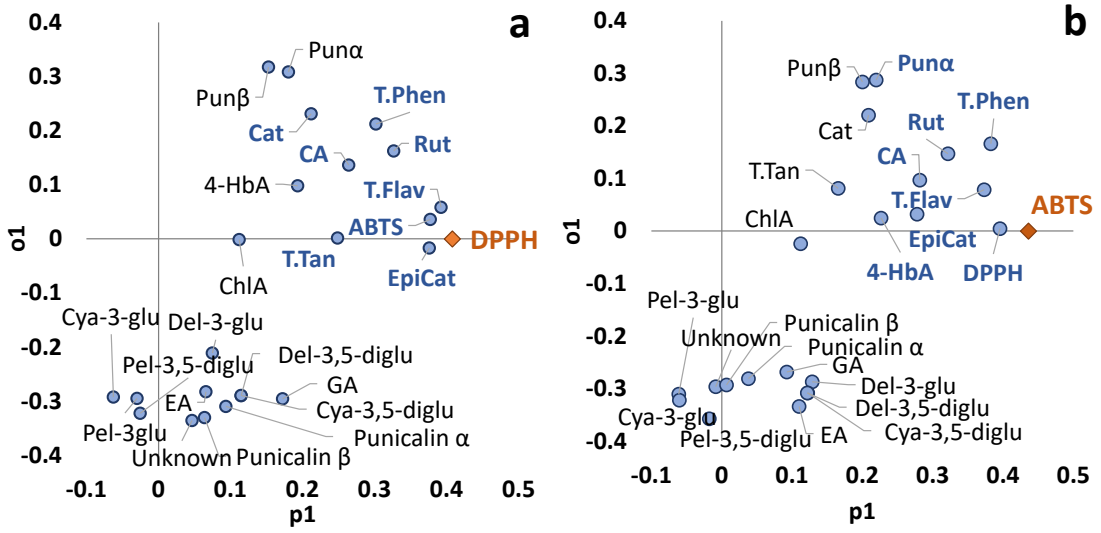

Figure 1 *OPLS loading scatter plots representing the relationships between the X variables (PPE chemical composition) and the Y variable: DPPH (a) and ABTS (b). The variables highlighted in bold blue type are those strongly correlated with the Y variable (in terms of variable importance on projection: VIP).*

3.2 antimicrobial activity of PPE

The results showed that PPEs were able to inhibit biofilm development at concentrations below the MIC of the tested isolates (Fig. 2). Benslimane et al. (2020) reported the inhibition of biofilm formation by PPE for all the bacteria strains tested in their study, and the level of inhibition increased by increasing extract concentrations. In the present study, significant reductions were also obtained at much lower concentrations of water-extracted PPE, with more than 65% reduction in biofilm formation observed at a concentration of 3 to 0.75 mg/ml for Gram-positive bacteria strains. The antibiofilm activity of PPEs could be attributed to the presence of phenolic compounds such as punicalagin and ellagic acid, which may exert their effects through different mechanisms of action (Balaban et al., 2021a).

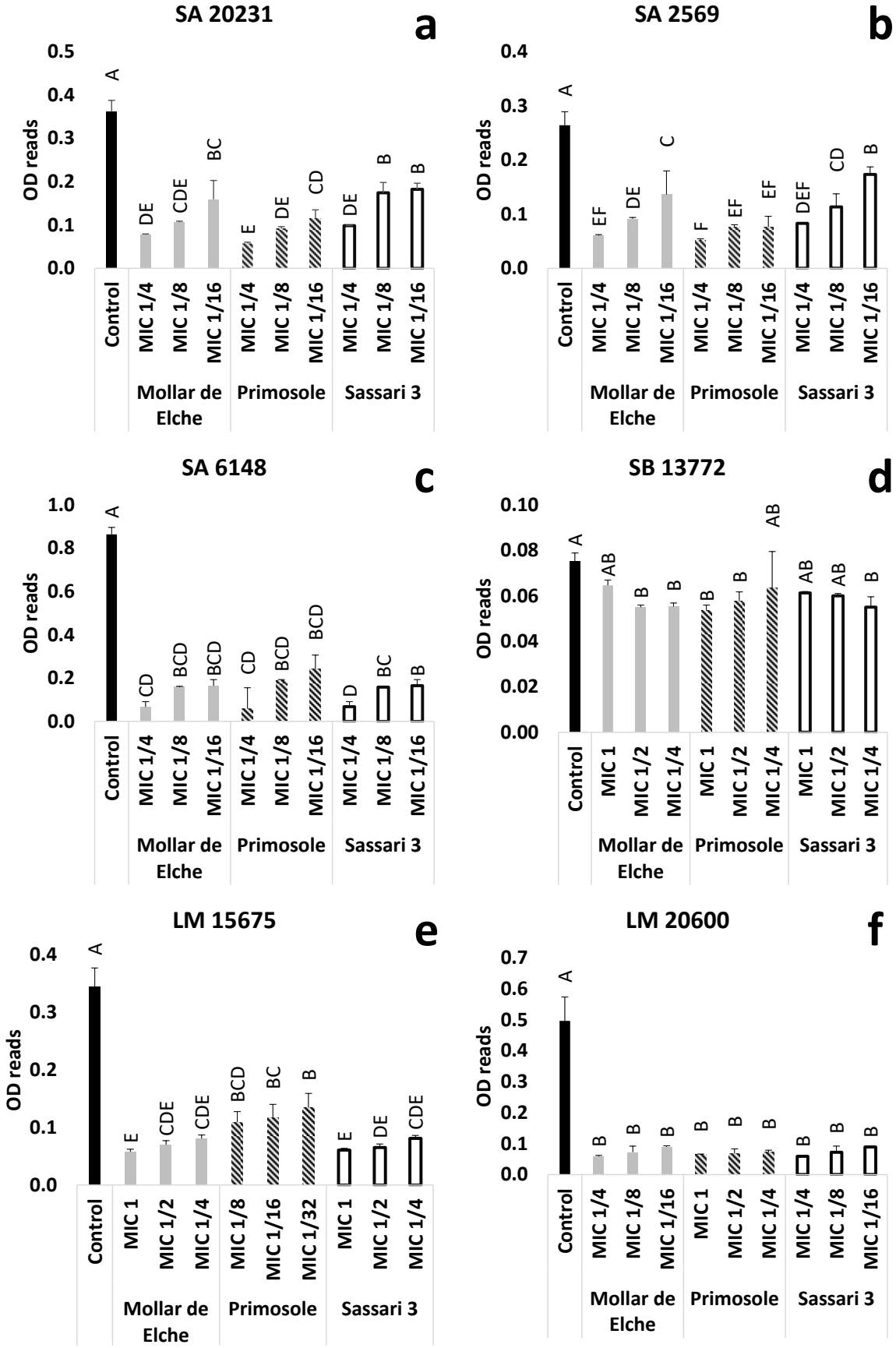
SA 20231
a
OD reads
0.5
0.4
0.3
0.2
0.1
0.0
A
DE
CDE
BC
E
DE
CD
DE
B
B
Control
MIC 1/4
MIC 1/8
MIC 1/16
MIC 1/4
MIC 1/8
MIC 1/16
MIC 1/4
MIC 1/8
MIC 1/16
Mollar de Elche
Primosole
Sassari 3
SA 2569
b
OD reads
0.4
0.3
0.2
0.1
0.0
A
EF
DE
C
F
EF
EF
DEF
CD
B
Control
MIC 1/4
MIC 1/8
MIC 1/16
MIC 1/4
MIC 1/8
MIC 1/16
MIC 1/4
MIC 1/8
MIC 1/16
Mollar de Elche
Primosole
Sassari 3
SA 6148
c
OD reads
1.0
0.8
0.6
0.4
0.2
0.0
A
CD
BCD
BCD
CD
BCD
BCD
D
BC
B
Control
MIC 1/4
MIC 1/8
MIC 1/16
MIC 1/4
MIC 1/8
MIC 1/16
MIC 1/4
MIC 1/8
MIC 1/16
Mollar de Elche
Primosole
Sassari 3
SB 13772
d
OD reads
0.10
0.08
0.06
0.04
0.02
0.00
A
AB
B
B
B
B
AB
AB
AB
B
Control
MIC 1
MIC 1/2
MIC 1/4
MIC 1
MIC 1/2
MIC 1/4
MIC 1
MIC 1/2
MIC 1/4
Mollar de Elche
Primosole
Sassari 3
LM 15675
e
OD reads
0.4
0.3
0.2
0.1
0.0
A
E
CDE
CDE
BCD
BC
B
E
DE
CDE
Control
MIC 1
MIC 1/2
MIC 1/4
MIC 1/8
MIC 1/16
MIC 1/32
MIC 1
MIC 1/2
MIC 1/4
Mollar de Elche
Primosole
Sassari 3
LM 20600
f
OD reads
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
A
B
B
B
B
B
B
B
B
B
Control
MIC 1/4
MIC 1/8
MIC 1/16
MIC 1
MIC 1/2
MIC 1/4
MIC 1/4
MIC 1/8
MIC 1/16
Mollar de Elche
Primosole
Sassari 3

Figure 2 *The effect of the three cold water PPE (from ME, PS, SS3 cultivars), applied at different concentrations (MIC[1]), on biofilm formation. Letters above the columns indicate statistical differences according to Tukey's test.*

4. Conclusions and Future Perspectives

The current study contributes to furthering our knowledge of the phenolic composition of pomegranate peel extracts in different national and international varieties, Expanding the study to include local varieties was important from the perspective of Italian plant breeding and the valorization of local biodiversity. Detailed characterization of the bioactive components of peel extracts from specific varieties of pomegranate is necessary in order to explain their antimicrobial, antibiofilm, and antioxidant activities against some of the most common pathogens. Three pomegranate varieties were shown to exhibit strong antimicrobial activity. Those varieties were shown to be rich in punicalagins, flavonoids, and chlorogenic acid, the presence of which can account for their antimicrobial activities. In conclusion, this study proposes a formulation of pomegranate peel extract that valorizes an agro-industrial waste in the context of sustainability and circular economy. Pomegranate extracts should be considered as potential sources of natural, plant-derived antimicrobials, providing an alternative to artificial antimicrobial products.

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Microbiota and metabolome in chronic non-communicable diseases

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This PhD thesis dealt with the study of the gut microbiota and metabolome in association with food and clinical aspects. Specifically, *i*) the relationship between intestinal microbiome and chronic non-communicable diseases (i.e., fructose intolerance, nephropathy, and obesity) were studied; *ii*) the use of probiotics in patients with fructose intolerance and nephropathy (Chronic Kidney Disease, CKD) was evaluated; *iii*) pasta samples contained bioactive waste ingredients were characterized and the antioxidant and microbiological activities were studied, for a possible application as functional food.

Microbiota e metaboloma in patologie croniche non trasmissibili

Questa tesi di dottorato ha riguardato lo studio del microbiota e del metaboloma intestinale in associazione ad aspetti alimentari e clinici. In particolare, *i*) è stata studiata la relazione tra il microbiota intestinale ed alcune patologie croniche non trasmissibili, come intolleranze alimentari, nefropatie e obesità; *ii*) è stato valutato l'impiego di probiotici in soggetti affetti da intolleranza al fruttosio e nefropatia (*Chronic Kidney Disease*, CKD); *iii*) dei campioni di pasta sperimentale contenente ingredienti di scarto bioattivi sono stati caratterizzati e studiati in termini di attività antiossidante e microbiologica, per una possibile applicazione come *functional food*.

Key words: intestinal microbiota; intestinal metabolome; chronic non-communicable diseases; functional food.

1. Introduction

In accordance with the PhD thesis project, this oral communication reports the main results of the following five activities directed to:
A1) Assess *i*) the prevalence of fructose intolerance (FI) in patients with FGIDs and *ii*) the effectiveness of the EQBIOTA probiotic in improving symptoms of FI.
A2) Explores the effect of the symbiotic NatuREN G on the gut microbiota of stage IIIb-IV CKD patients.
A3) Provide a comprehensive view of the correlation between the intestinal microbiome and the metabolic alterations in obese patients.
A4) Investigate the differences of the gut microbiota and metabolome in patients affected by different types of nephropathies.
A5) Characterize and analyse the microbiological activity of functional pasta samples enriched with different bioactive waste ingredients.

2. Materials and Methods

2.1 Assess *i*) the prevalence of FI in patients with FGIDs and *ii*) the effectiveness of the EQBIOTA probiotic in improving symptoms in fructose intolerants.

The prevalence of FI in a cohort of Romanian adult with Functional Gastrointestinal Disorders (FGIDs) and the effectiveness of treatment with a new probiotic formulation EQBIOTA™ (*Lactiplantibacillus plantarum* CECT 7484 and 7485 and *Pediococcus acidilactici* CECT 7483) were evaluated. FI subjects on fructose-free diet regimen and persistent symptomatology and healthy volunteers (HC) tested the probiotic for 30 days. The gastro-intestinal symptoms (abdominal pain and bloating), bowel habits, and fecal volatile metabolome were evaluated before (T0) and after treatment (T30). The Volatile Organic Compounds (VOCs) were quantified using the gas chromatography coupled by mass spectrometry (GC-MS).

2.2 Explores the effect of the symbiotic NatuREN G on the gut microbiota of stage IIIb-IV CKD patients.

The effect of symbiontic (S) NatuREN G® (*Bifidobacterium animalis* BLC1, *Lacticaseibacillus casei* LC4P1, fructo-oligosaccharides, inulin, quercetin, resveratrol, and proanthocyanidins) on gut microbiota in a single-blind, placebo-controlled, pilot trial, involved both CKD patients at IIIb-IV nephropathy-stage and healthy controls (HC), was evaluated. The placebo (P) used in the present study was based on maltodextrins and aromas. Faecal samples and dietary questionnaires were collected at the beginning of the study (T0), after 60 days of treatment (T60), and after further 30 days of wash out (T90). Fecal samples were analyzed by 16S rDNA metataxonomics and GC-MS to evaluate metabolic profile. Finally, changes in *Lactobacillus* and *Bifidobacterium* genera were also assessed by

quantitative PCR.

2.3 Provide a comprehensive view of the correlation between the intestinal microbiome and the metabolic alterations in obese patients.
The aim of this study was to characterize gut microbiota and metabolome composition in metabolically healthy (MHO) and unhealthy (OB) obese adult subjects considering their clinical data and dietary conditions. An observational study was conducted on healthy controls (HC) and MHO and OB subjects. Blood samples and 3 days food questionnaires were collected. The gut microbiota was characterized in faecal samples through quantitative PCR with the use of primers for specific bacterial genera and species. GC-MS was performed for the analysis of untargeted metabolites and short-chain fatty acids in faecal samples.

2.4 Investigate the differences of the gut microbiota and metabolome in patients affected by different types of nephropathies.
The impaired kidney function in nephropathic patients was linked to an unbalanced microbiota asset. To better detail the reasons of this dysbiosis, an observational study was performed to investigate the gut microbiota and volatile metabolome in four kidney pathologies: Chronic Kidney Disease (CKD), Diabetic Kidney Disease (DKD), Autosomal Dominant Polycystic Kidney Disease (ADPKD), and Immunoglobulin A nephropathy (IgAn). A healthy control group (HC) was added to the study. Blood samples were collected to analyse the biochemical parameters. Moreover, subjects provided faecal samples to investigate the gut microbial population through quantitative PCR and the volatile metabolome through GC-MS.

2.5 Characterize and analyse the microbiological activity of functional pasta samples enriched with different bioactive waste ingredients.
Experimental samples of pasta were produced by the Casillo Next Generation Food Group (Corato, Italy). Pasta was enriched with three different ingredients: i) deoleated durum wheat germ; ii) deoleated durum wheat bran; iii) durum wheat oil. By combining the three ingredients, four samples of pasta were formulated: dry pasta with 30% deoleated durum wheat germ (GP); dry pasta with 30% deoleated durum wheat bran (BP); dry pasta with 27% deoleated durum wheat germ and 6% microencapsulated durum wheat oil (GPmO); dry pasta with 27% deoleated durum wheat bran and 6% microencapsulated durum wheat oil (BPmO). Dry pasta obtained from semolina with the addition of integral-like dye was used as control (CP).
In vitro tests were performed in order to: i) assess the antioxidant activity and the content of phenols (according to Difonzo et al., 2017 and Limongelli et al., 2023) of pasta extracts, and ii) characterize the microbiological activity of fermented digested pasta samples. Digestion was simulated *in vitro* using enzymes from oral, gastric, and intestinal fluids (De Angelis et al., 2021). Samples of digested pasta were fermented *in vitro*, simulating the colonic fermentation, according to Vacca et al. (2023). For the microbiological activity, some target genera and species were investigated through quantitative PCR and an aliquot of each fermented sample supernatant was analyzed to characterize the Volatile Organic Compound (VOC) through GC-MS.

3. Results and Discussion

3.1 Assess *i*) the prevalence of FI in patients with FGIDs and *ii*) the effectiveness of the EQBIOTA probiotic in improving symptoms in fructose intolerants.
The prevalence of fructose intolerants (31.8%) within FGID group was higher than lactose intolerant subjects (6.8%). The final group of enrolled patients consisted of 14 FI and 13 HC subjects. In FI group, the values of gastro-intestinal symptoms (VAS) were significantly higher than HC. Treatment with EQBIOTA caused an overall improvement of symptoms in FI subjects: we observed a significant decrease of bloating (q = 0.0001) and abdominal pain (q = 0.0002).

Table 1. *Baseline characteristics expressed as mean ± SD and median values of the fructose intolerant and healthy control group enrolled for EQBIOTA treatment.*

	Healthy control			Fructose intolerant		
	T0	T30	*p*-value	T0	T30	*p*-value
Bristol Score (BSFS)	3.3 ± 0.5	3.6 ± 0.4	n.s.	2.9 ± 1.1	3.4 ± 0.5	n.s.
Bloating (VAS, mm)	6.9 ± 11.8	11.2 ± 6.5	n.s.	68.6 ± 21.4	13.6 ± 17.8	p = 0.0001
Abdominal pain (VAS, mm)	2.3 ± 4.4	5.9 ± 3.1	n.s.	43.6 ± 28.7	7.1 ± 13.3	p = 0.0002

118 VOCs were identified and grouped according to chemical classes. The content of the VOCs largely varied within samples, some significant differences were evaluated by comparing HC and FI group at the T0, and at the

T30 in FI group. The permutation analyses showed how the VOCs profile of HC-T0, HC-T30, and FI-T30 were grouped in one single cluster, whereas fructose intolerant group at the T0 was un-clustered. In the FI-T30 group, some Medium Chain Fatty Acid (MCFA) resulted higher compared to FI-T0, specifically hexanoic acid and heptanoic acid. Interestingly, the MCFA are resulted discriminant between healthy subjects and patients with gastrointestinal pathologies (De Preter et al., 2015).
A correlation analysis was performed between fecal metabolome and clinical symptoms. The bloating scores in fructose intolerant subjects was negatively correlated with 1-pentanol ($r = -0.60$, $q = 0.03$), hexanoic acid ($r = -0.53$, $q = 0.04$), and carvacrol ($r = -0.59$, $q = 0.03$). The abdominal pain score showed the same trend, in detail showed a negative correlation with 1-pentanol ($r = -0.50$, $p < 0.05$), carvacrol, and beta-bisabolene ($r = -0.50$, $p < 0.05$). Conversely, the ethyl ester of hexadecenoic acid was positive correlated with bloating ($r = 0.54$, $p < 0.05$) and abdominal pain ($r = 0.60$, $q = 0.01$) scores. This compound belongs to the Fatty Acid Ethyl Esters (FAEE). It has been shown that FAEE are able to induce dysfunctions of the intestinal barrier, with the induction of oxidative stress at the level of the intestinal *epithelium* (Elamin et al., 2013)
The treatment with EQBIOTA determined changes in VOC profile of FI patients with a specific increase of potentially anti-inflammatory and protective compounds. These changes occurred in parallel with a decrease of FAEE, which might have a potential detrimental effect on the intestinal barrier and oxidative stress.

3.2 Explores the effect of the symbiotic NatuREN G on the gut microbiota of stage IIIb-IV CKD patients.
The alpha diversity of the GI microbiota reported no differences comparing the run-in (T0) values after the randomization (P *vs* S) of both CKD and HC. NatuREN G® increased the number of identified species in both subgroups (CKD and HC), but the carry-on effect till T90 was found only in CKD. In CKD-S, NatuREN G® significantly shifted the ratio Firmicutes/Bacteroidetes. In detail, Firmicutes were positively affected by the NatuREN G® being found significantly associated to the innovative synbiotic at both follow-ups, whereas Bacteroidetes showed the opposite. At family level, *Coriobacteriaceae* and *Flavobacteriaceae* positively and negatively correlated with the NatuREN G®, respectively, since these tendencies were also detected till the end of the trial (T90). *Blautia* was the only genus that showed a positive correlation with NatuREN G® at both follow-ups. According to 60 days of treatments, only *Selenomonas* significantly differed in CKD patients between subgroups (P *vs* S) in metataxonomic relative abundances. Specifically, the taxa had a higher relative abundance in CKD treated with synbiotic than treated with probiotic too. At T60 and T90 not significantly increased in numbers for *Lactobacillus* were observed in CKD-S, while decreased in CKD-P ($p < 0.05$). In CKD-S, the abundance of *Bifidobacterium* increased during the study. The metabolic profiles varied between groups after treatment. The synbiontic NatuREN G® was able to modulate the gut microbiota profiling and the related metabolism in stage IIIb-IV CKD patients. Specifically, NatuREN G® increased the ratio Firmicutes/Bacteroidetes and this was reflected in an increase of the saccharolytic metabolism reducing the proteolytic one, according with an increased concentration of acetic and propanoic acids in faecal samples. Therefore, present work opens the way towards further studies based on nutritional managements and adjuvant therapies based on probiotics and prebiotics administration in diseases, such as the nephropathy.
3.3 Provide a comprehensive view of the correlation between the intestinal microbiome and the metabolic alterations in obese patients.
The clinical picture of OB patients differed from HC and MHO ones. Compared to MHO, OB patients showed higher levels of HOMA index ($q = 0.01$) and Glycate Haemoglobin ($q = 0.03$), and lower level of Glomerular Filtration Rate ($q = 0.03$). The DAPC statistical analysis reported the separation of subjects in three groups. Several variables have a greater impact on the subdivision of the groups, including body mass index (BMI), erythrocytes sedimentation rate (ESR), C reactive protein (CRP), and several thyroid hormones. A target group of intestinal population were investigated (Fig. 1). The amount of *Clostridium coccoides* was lower in OB and MHO than in HC group ($q = 0.0003$). Studies reported the same trend of *Clostridium* species comparing obese and control subjects, and *Clostridium coccoides* resulted inversely related to insulin and HOMA index levels (Teixeira et al., 2013). *Lactobacillus* genus ($q = 0.02$) and *Lactiplantibacillus (Lp.) plantarum* ($q = 0.02$) were higher in MHO subjects, while *Prevotella* ($q = 0.03$), *Desulfovibrio* ($q = 0.02$), and *Lp. plantarum* ($q = 0.04$) enriched the microbiome of OB patients, compared to HC. *Prevotella* is correlated with the hormone ghrelin, lead to an increment of appetite (Gomes et al., 2018); while higher amounts of *Desulfovibrio*, is correlated with metabolic alteration, as Non Alcoholic Fatty Liver Disease (Lin et al., 2022). Considering volatile metabolome, compared to MHO subjects, the HC group presented higher quantity of tetradecane, 2H-indol-2-one-1,3-dihydro, 2-tridecanone, benzeneacetaldehyde, butanal-3-methyl-2, and gamma-terpinene ($p > 0.05$). Compared to OB subjects, HC volunteers reported greater amount of nonanoic acid, gamma-terpinene, cyclohexanecarboxylic acid, pentanoic acid, butyl ester, alpha phelladrene, and humulene. On the other hand, OB subjects showed higher levels of 2-undecanone, 2-pentadecanone, and 2-hexadecanone compared to HC subjects, and higher levels of nonadecane, indole, and 1H-pyrrole-2,5-dione, 3-ehyl than MHO group. In addition, a lower presence of butanoic acid was observed in OB patients compared to MHO group. The preliminary results support the association between metabolic diseases and differences in gut microbiota between healthy and unhealthy obese subjects. Furthermore, such differences in gut microbiota could be used as biomarkers for a less invasive diagnosis of pathological obesity.

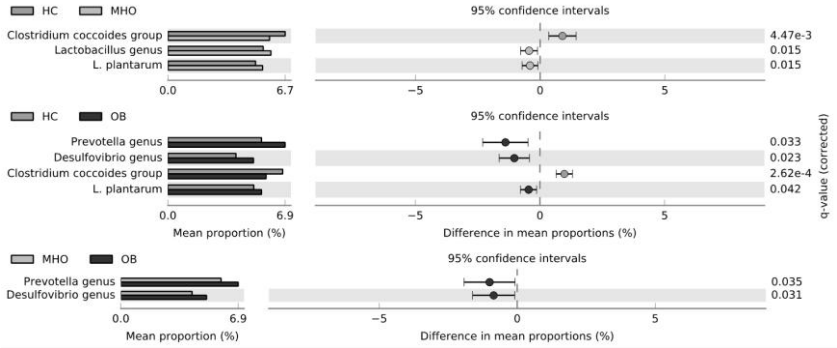

Figure 1. *Statistically significant qPCR tested taxa emerging from pairwise comparison of HC, MHO, and OB cohorts.*

3.4 Investigate the differences of the gut microbiota and metabolome in patients affected by different types of nephropathies.

Blood biochemical variables highlighted four distinct profiles relative to the four groups of nephropathic patients. A discriminant analysis of principal components separated samples based on the microbial profiles. In detail, HC and ADPKD were separated to CKD, DKD, and IgAn groups, which clustered together (Fig. 2).

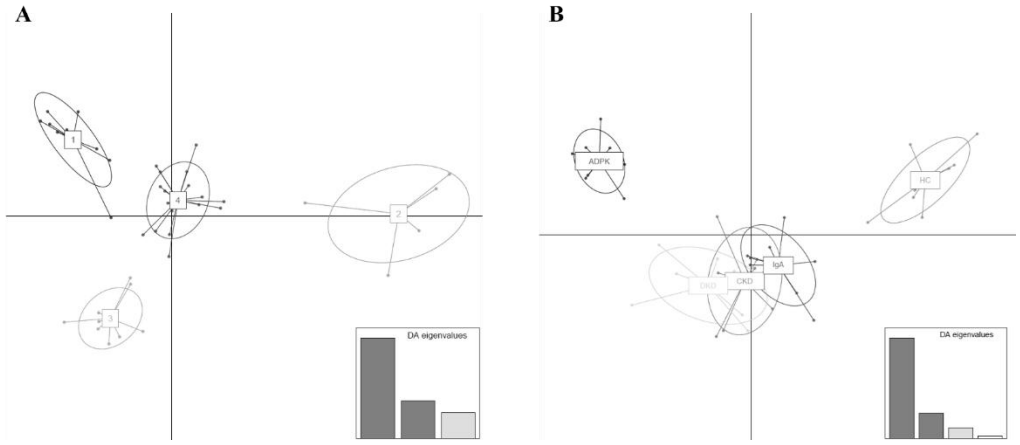

Figure 2. *A priori (A) and a posteriori (B) DAPC analysis of qPCR analysis.*

The variables that had the main impact on the subdivision in groups include species belonging to *Bifidobacteria*, *Lactobacillus*, *Bacteroides*, and *Clostridium*, and *Bifidobacterium*, *Prevotella*, *Desulfovibrio*, and *Atopobium* at the genus level. The analysis of volatile metabolome led to distinguish three clusters of samples. IgAn was characterized by greater amounts of esters and ketones, ADPK and CKD by aldehydes, terpenes, sulfuric compounds, and fatty acids, while DKD and HC by greater concentration of indoles, hydrocarbons, alcohols, phenols, and carboxylic acids.

The different nephropathies were correlated with altered homeostasis. Our results highlighted the differences of microbial population and intestinal metabolome associated with the different impaired kidney functions. These preliminary results could be useful to understand the role of gut microbiota in nephropathic diseases, and evidence the possibility of identifying some metabolites and taxa as biomarkers useful to predict and stratify patient groups.

3.5 Characterize and analyse the microbiological activity of functional pasta samples enriched with different bioactive waste ingredients.

From the analysis of the pasta extracts, the samples subdivision in three groups based on their characteristics (phenols quantity and antioxidant activity) has emerged. In detail, GPmO and GP samples were included in the group with the highest levels of antioxidant activity and phenols quantity; the second group included BPmO and BP samples, characterized by intermediate antioxidant activity and phenolic content; finally, CP sample was excluded from the other groups, with the lowest levels of measured parameters. *In vitro* digestion results highlighted the impact of the pasta tested on the intestinal microbial population (Fig. 3). Coliforms, streptococci, total aerobes microbial count (TAMC), and total anaerobes microbial count (TANMC) were mainly present in

GPmO sample. Lactic acid bacteria (LAB) population was significantly underrepresented in GPmO and BPmO samples. GP and BP non-microencapsulated samples showed a greater population of bifidobacteria than the others.

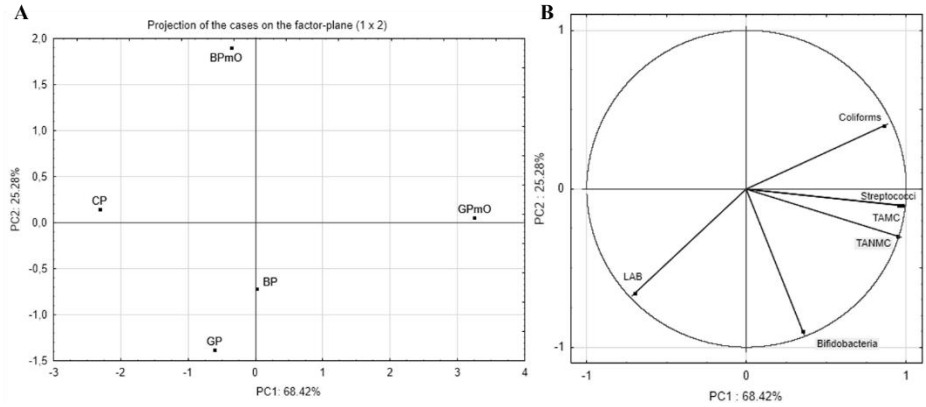

Figure 3. *Results on cell viability in fermented samples. The first graph (A) shows the arrangement of cases (samples of pasta); the loading plot (B) shows the cell densities of the plated microbial groups.*

Based on the analysis of a target group of microbial population, we observed differences between the five samples of pasta, each characterized by a microbial profile. Based on statistically different compounds, a hierarchical clustering analysis was performed. BPmO un-clustered with the other samples. The other two groups included CP and BP samples in the first and GPmO and GP samples in the other one. Finally, a targeted analysis of Short Chain Fatty Acid (SCFA) was conducted. GPmO sample presented the highest quantities of all SCFA and showed statistically significant ($p < 0.05$) higher amounts of isovaleric acid and isobutyric acid, compared both to CP, GP, and BPmO samples, and higher quantities of butanoic acid and propanoic acid, compared to BPmO and CP samples, respectively. In summary, the analyses carried out reported different antioxidant and microbiological characteristics of the experimental pasta preparations, based on the added ingredients.

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Optimization of the extraction techniques using Natural Hydrophobic Deep Eutectic Solvents for the recovery of biomolecules from food and food industry by-products

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This PhD Project focused on the optimization of a green extraction technique based on Natural Hydrophobic Deep Eutectic solvents (HDESs) for the recovery of carotenoids from by-products of the vegetable food processing industry as well as an unconventional source, as algae. The implemented experimental design was structured in three steps: 1) assessment of the physicochemical properties and the extracting efficiency of several Natural HDESs for the recovery of carotenoids from different substrates; 2) selection of the best performing solvents and substrates and optimization of the extraction technique; 3) development of food and cosmetic applications of the enriched carotenoids extracts evaluating the antioxidant stability and the consumers' acceptability of the obtained new products.

Ottimizzazione delle tecniche di estrazione mediante Natural Hydrophobic Deep Eutectic Solvents per il recupero di biomolecole da prodotti e sottoprodotti dell'industria alimentare

Questo progetto di Dottorato di Ricerca è stato focalizzato sull'ottimizzazione di una tecnica di estrazione verde utilizzando solventi eutettici profondi idrofobici (HDESs) per il recupero di carotenoidi da sottoprodotti vegetali dell'industria alimentare e da una fonte alimentare non convenzionale quale le alghe. Il progetto è stato strutturato in tre fasi: 1) valutazione delle proprietà chimico fisiche e dell'efficienza estrattiva di diversi HDES naturali; 2) selezione dei solventi e dei substrati più performanti e ottimizzazione della tecnica di estrazione; 3) sviluppo di applicazioni alimentari e cosmetiche degli estratti arricchiti di carotenoidi, valutandone la stabilità antiossidante e l'accettabilità da parte dei consumatori.

Key words: food by-products, green extraction, microalgae, natural hydrophobic deep eutectic solvents, optimization.

1. Introduction

In recent years, sustainability and green engineering principles have been the ground for scientific research in many field. In the food sector, the development of sustainable and economically viable bio-based processes to obtain highly added-value compounds for functional foods and dietary supplements production is a hot topic, due to the increasing consumers' awareness of the pivotal role played by nutrition in human health. Furthermore, also pharmaceutical and cosmetic industries have an interest in new moieties, which may be utilized in product formulation.

Table 1. *Structure of this PhD project.*

Aim	Activities
Implementation of green extraction processes for carotenoids recovery	• Selection of suitable Natural HDESs according to the literature review; • Selection of rich-carotenoid matrices; • Physicochemical characterization of the selected Natural HDESs; • Selection of the best performing solvents for each matrix.
Optimization of the extraction process by implementing a Box-Benkhen Design with the goal of maximizing carotenoid yield in the extracts*	• Identification of the proper combination between HBA:HBD molar ratio, solvent to sample ratio and the optimum extraction time; • HPLC analysis on the extracts; • Design of a purification step.
Development of food and cosmetic applications for the enriched carotenoids extracts**	• Production of cosmetic products and food supplements added with the obtained extracts; • Antioxidant stability and consumer tests.

** ongoing activities ** activities to be conducted in the last semester of this PhD project.*

Nowadays, the industrial recovery of these moieties represents a challenging step, with problems related to costs, efficiency, selectivity and environmental sustainability (Choi et al., 2019). About this latter issue, the use of Natural Deep Eutectic Solvents (NaDESs) has proved to be a potential alternative for the green extraction of natural bioactive compounds (Cvjetko Bubalo et al., 2018; Sportiello et al., 2023). NaDESs represent a subcategory of the Deep Eutectic Solvents (DESs), which are peculiar mixtures obtained by combining two or more constituents, generally solid at room temperature, with a resultant melting point depression and the transition into a liquid state. DESs can be easily prepared by mixing hydrogen bond acceptors (HBAs) and hydrogen bond donors (HBDs) in specific molar ratios (Martins et al., 2018). NaDESs are obtained when limiting the selection of the HBA and HBD to moieties derived from natural sources and, depending on the resultant polarity of the mixture, they can be hydrophilic or hydrophobic, thus able to solubilize an extensive range of molecules. Monoterpenes, carboxylic and fatty acids are the most common natural HBAs and HBDs utilized for realizing Natural Hydrophobic DESs (HDES), generally having a very low cost and negligible ecological impact and toxicity. Additionally, their high biocompatibility and their food-grade nature open the way for new direct applications of the extracts *'as such'* in the food, cosmetic and pharmaceutical industry. Based on these assumptions, the aim of this PhD project is the optimization of a green extraction technique using Natural HDESs for the recovery of carotenoids from matrices of emerging interest, such as microalgae, and several real vegetable by-products supplied by industries located in Northeast Italy. Additionally, a further aim is the formulation of products enriched with the obtained extracts, demonstrating the potential use in industrial applications. In Table 1 are reported the activities carried out to achieve the goals of this PhD project.

2. Materials and Methods

2.1 Sample and solvent preparation and physicochemical characterization

Initially, as extraction substrates were utilized by-products (peels) deriving from the industrial processing of fresh carrots, yellow and red peppers and pumpkins, and were kindly supplied from Ortonuovo Srl (Arbizzano-Santa Maria, VR). The collected samples were cleaned, comminuted, freeze-dried and stored at -20 °C until use. In a second step of the experimental research, lyophilized samples of the microalga *Chlorella vulgaris*, another carotenoid-rich substrate, were tested. All samples were characterized for their water content and water activity (a_w). Natural HDESs were prepared according to the method proposed by Dai et al. (2014), with slight modifications. The two solid components in pre-set molar ratios were placed in a bottle with a stirring bar and cap and heated in a water bath at 70 °C for 30-60 min, till a clear liquid was formed. For the carotenoid extraction from plant by-products, eleven Natural HDESs were prepared utilizing monoterpenes (camphor and thymol) as hydrogen bond acceptors and carboxylic acids (lactic and decanoic acids) as hydrogen bond donors; furthermore DL-menthol was utilized as both HBA and HBD. For the extraction from *Chlorella vulgaris* biomass, other seven natural HDESs were prepared using fatty acids (caprylic, pelargonic, capric acid and lauric acids) as HBAs and HBDs. All natural HDESs were physicochemically characterized assessing their a_w, density (γ) and dynamic viscosity (μ). Furthermore, the Natural HDESs' density was assessed in the temperature range 20 - 60 °C andthe viscosity in the temperature range 20-60 °C and applying different shear-rates, ranging from 50 to 300 s^{-1}.

2.2 Assessment of the extraction efficiency

Preliminary extraction tests were performed adding 0.1 g of lyophilized sample to 5 mL of each Natural HDES (sample:solvent 1:50). The mixture was vortexed at 25 °C for 60 s and then kept under continuous mixing for 30 min using a rotating mixer Afterwards, the sample was sonicated for 60 min at 45 kHz, before being centrifuged at 3900 RCF for 10 min. The amount of carotenoids extracted was assessed by spectrophotometric measurement, taking readings at 450 nm, as reported by Scott (2001). For the extraction using plant by-products as substrates, a given aliquot of the supernatant was diluted with acetone (1:5) taking the absorbance at 450 nm and the carotenoid content was quantified as β-carotene, while, for the microalga matrix, a higher dilution with acetone (1:50) was utilized. The extracting trials were performed in triplicate. As a reference, the extractions were carried out also with acetone, an organic solvent that finds use in the recovery of carotenoids from vegetable matrices for food purposes. Statistical analysis of the data was performed using the software XLSTAT Premium (Version 2020.3.1, Addinsoft, Paris, France). Data were analyzed by one-way ANOVA and significant differences among means were computed by Tukey's HSD test (Honestly Significantly Different) at a significance level of 0.05.

Optimization of the extraction processes

The selection of **the best** performing Natural HDESs for the extraction from the four plant by-products was achieved based on the results of the preliminary extraction tests. Afterward, the optimum conditions for maximizing the extraction efficiency were evaluated by implementing a three-factor, three-level Box–Behnken experimental design (BBD) combined with response surface modeling (RSM). For this study, the effect of HBD:HBA molar ratio (x1), solvent to sample ratio (x2) and extraction time (min, x3) were selected as independent variables and studied at three different levels coded as -1, 0 and +, with 5 central points, for a total of 17 runs. In order to obtain a more robust data set, each run, except the central ones, was carried out twice, for a total of 29 experiments for each investigated HDES. The response variables selected to be optimized were β-carotene and lutein yields (y1 and y2), which were separated on a, C30 column (4.6 × 250 mm, 5 μm, YMC Inc.,

Wilmington, NC) using a HPLC–diode array detector system. Peaks were separated by gradient elution according to the procedure described by Stupar et al., (2021). With regard to the selected best extracting Natural HDES for the extraction from *Chlorella vulgaris* substrate, the process optimization using the same design of experiment is ongoing. The statistical analysis was carried out using the software Design Expert software (Version 8.0.7.1, Stat-Ease Inc., USA). The optimized values of the three factors were obtained using the software's desirably function, with values ranging from 0 (completely undesirable response) and 1 (fully desirable response).

3. Results and Discussion

3.1 Natural HDESs preparation and physicochemical characterization

Natural HDESs have been reported to possess efficient extraction capabilities towards carotenoid compounds present in foods (Silva et al., 2019; Stupar et al., 2020). In this research, several HDESs were prepared and tested as valuable solvents for the recovery of carotenoids from different matrices. As showed in Table 2, eighteen Natural HDESs were prepared combining different starting materials in specific molar ratios. Eleven (from HDES 1 to HDES 11) were tested for the extraction of the carotenoid fraction from carrot, yellow and red pepper and pumpkin peels, while seven (from HDES 12 to HDES 18) were used for the extraction using a microalgae, *Chlorella vulgaris*, as the substrate. A first selection was made observing the solvent stability during storage after their preparation. Actually, HDES 3, 8 and 11 showed thermal instability with tendency to separate when cooled below 25 °C, giving rise to two layers and requiring subsequent heating for their use as an extraction media. Therefore, they were excluded from the subsequent investigation steps. Furthermore, also HDES 17 showed instability when the room temperature dropped at 20 °C, but, due to the easy restore of the liquid state at already 23 °C, the solvent was investigated with the others taking care of maintaining the working temperature at 25 ± 1 °C.

Table 2. *Composition and physical characteristics* of the investigated Natural HDESs.

Natural HDES	HBA/HBD	Molar ratio	Density (g/cm^3 at 25 °C)	Viscosity* (mPa·s at 25° C)
HDES 1	DL-menthol/lactic acid	1:1	0.981	56.71
HDES 2		1:2	1.031	54.82
HDES 3		8:1	0.898	134.68
HDES 4	DL-menthol/decanoic acid	1:1	0.894	35.24
HDES 5		6.5:3.5	0.921	31.78
HDES 6	thymol/DL-menthol	1:1	0.935	37.86
HDES 7		1:2	0.924	54.68
HDES 8	thymol/decanoic acid	1:1	n.a.	n.a.
HDES 9		3:2	0.919	18.86
HDES 10	camphor/decanoic acid	1:2	0.931	25.58
HDES 11		1:1	n.a.	n.a.
HDES 12	caprylic acid/capric acid	2:1	0.900	9.841
HDES 13		3:1	0.901	9.642
HDES 14		4:1	0.863	8.786
HDES 15	caprylic acid/lauric acid	3:1	0.901	12.59
HDES 16	pelargonic acid/lauric acid	3:1	0.858	15.56
HDES 17	capric acid/lauric acid	2:1	0.892	17.12
HDES 18	pelargonic acid/capric acid/lauric acid	3:1:1	0.896	13.58

* measured at shear rate 50 s^{-1}; n.a. = not assessable.

Table 3. *Power law model: n and k values calculated for the different Natural HDESs selected.*

	Natural HDESs tested on vegetable by-products							
	HDES 1	HDES 2	HDES 4	HDES 5	HDES 6	HDES 7	HDES 9	HDES 10
n	0.70	0.73	0.45	0.68	0.67	0.75	0.70	0.66
k	149.62	142.19	135.24	83.73	173.01	107.39	89.25	182.76
	Natural HDESs tested on microalgae							
	HDES 12	HDES 13	HDES 14	HDES 15	HDES16	HDES17	HDES18	
n	0.65	0.66	0.81	0.65	0.64	0.78	0.78	
k	93.51	73.82	8.15	210.92	306.79	84.76	46.32	

n = power law index; k = consistency index

The density and the viscosity values assessed for the various solvents prepared are reported in Table 2. Density ranged from 0.858 to 1.031 g/cm^3 at 25 °C, with HDES 16 and HDES 2 showing the lowest and the highest

density values, respectively. As far as viscosity, the obtained values showed how all the Natural HDESs, except HDES 3 (excluded for its instability), fulfil one of the four standards established to assess the sustainability of these solvents from a chemical engineering point of view, namely a viscosity smaller than 100 mPa·s (van Osch et al., 2020). Furthermore, as reported in the previous section, the Natural HDES viscosity was assessed in the shear rate range 50 – 300 s^{-1}. This investigation was carried out in order to acquire information on the rheological flow behavior of these solvents and the data obtained show that the eighteen HDESs are non-Newtonian fluids. In particular, they have a shear-thinning behavior, with a decrease in the viscosity when higher values of shear rate are applied. This assumption was substantiated by the "n" values obtained when using the Power Law Model (Table 3).

3.2 Assessment of the extraction efficiency

Of the initial 18 natural HDES prepared, 8 were tested for the extraction of carotenoids from vegetable peels (HDES 1-2, 4-7, 9-10) while the remaining 7 were tested for the extraction of the compounds of interest from *Chlorella vulgaris* (HDES 12-18). Their composition is reported in Table 1. The extraction recoveries, calculated as percentage of the extraction yield obtained by using acetone, were statistically evaluated by using ANOVA in order to assess differences and to identify the solvent(s) suitable to be further investigated (Figure 1).

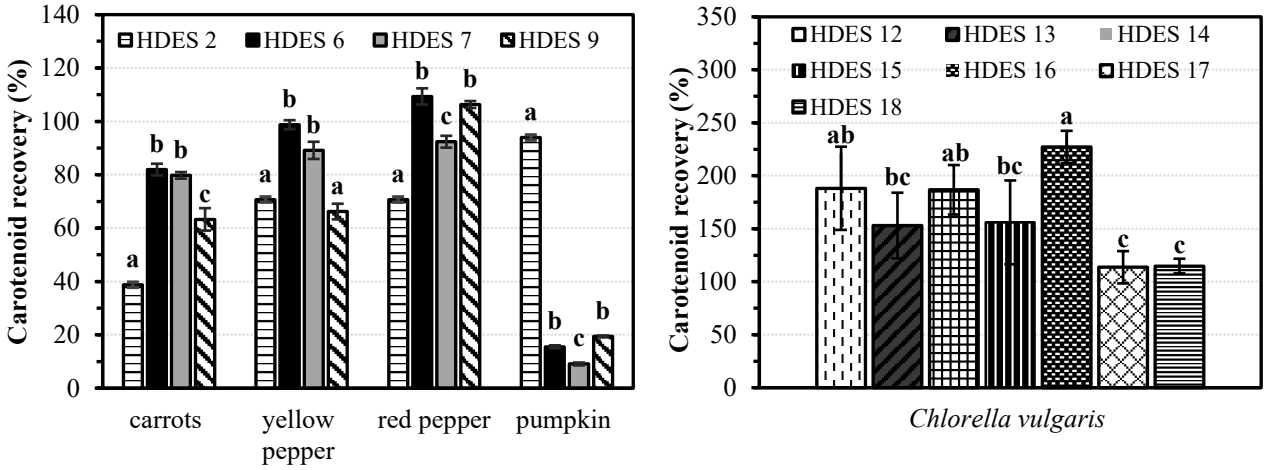

Figure 1. *Percentage of carotenoid recovery from different matrices using different Natural HDESs with reference to acetone extraction. For each matrix, values with different letters are significantly different for p<0.05.*

As far as the four different vegetable matrices, the HDESs utilized allow to achieve recoveries higher than 80% for 2 out of 4 of the tested substrates, with values near or over 100% when working on yellow and red pepper. In the case of the carrot, the recovery was still high, but only around 80% of the potentially allowable carotenoids were extracted using HDES 6 and 7. Generally speaking HDES 6 and 7 allowed to obtain the best results, except when working with pumpkin skin, In this case the highest recovery was obtained by using HDES 2, about 95%, while for all the other DES the percentage was very limited, not reaching the 20% value. Surprisingly, the HDES 7 showed the worst performance, with just a 9% recovery. Actually, HDES 6 and 7 were both prepared with thymol/DL-menthol at different molar ratios, and the increase of the DL-menthol amount, going from 1:1 to 1:2 as molar ratio negatively influenced the extraction percentages. Taking into account the above reported results, the HDES 6 was chosen to be further investigated when working with carrots and yellow pepper, while HDES 2 was chosen to be tested working on pumpkin skins. For red pepper by-products, the choice was oriented to the use of the HDES 9, because even if the assessed recovery was slightly less than that obtained with HDES 6 (106 % *vs* 109%) the data were not significantly different at $p<0.05$. Furthermore, working with a DES made up with thymol/decanoic acid rather than thymol/DL-menthol was considered a potentially positive aspect due to the lower cost of the solvent and its less intense menthol aroma.

With regard to the extraction from the microalga, very encouraging results were achieved, since the extraction efficiency ranged from 114 to 227 % with respect to the acetone extraction at the same operating conditions. The ANOVA analysis allowed identifying significant differences among the various solvents (Figure 1), with HDES 12, 14 and 16 giving the highest recoveries. The worst extraction performances were recorded when using capric acid and lauric acid as HDES constituents (HDES 17 and 18). On the basis of the obtained results, and being HDES 12, 13 and 14 realized with the same components, but at a different molar ratio, it was decided to continue the investigation optimizing the extraction process with reference to the composition of the HDES 16 and on the use of caprylic acid/capric acid as the HDES components.

3.3 Optimization of the extraction processes

In order to identify the best extraction conditions, a BBD was utilised as previously described. In Table 4 are

reported the values of the independent variables utilised to optimise the extraction process. In total 116 experiments were carried out and the resulting data were utilised to optimise the recovery of the carotenoid fraction from each substrate. The estimated optimal extraction conditions were as follows: solvent/sample ratio 50 for all the substrates; HBA/HBD ratio 2.5:1 for carrots, 5.75:1 for yellow pepper, 1.95:1 for red pepper, 4.68:1 for pumpkin skins; extraction time 30 min for carrots and red pepper, 76 min for yellow pepper, and 90 min for pumpkin skins. Practical validation of the model is actually undergoing, as well as the extraction optimisation of the carotenoid fraction from *Chlorella vulgaris* with the selected Natural HDESs.

Table 4. *Natural HDES Extraction optimization: values of the independent variables for recovering the carotenoid fraction from vegetable by-products.*

Independent variables	Levels		
	(-1)	(0)	(1)
HBA:HBD molar ratio			
HDES 6 (carrot and yellow pepper)	0.25	4	7.75
HDES 9 (red pepper)	0.5	1.50	2.50
HDES 2 (pumpkin skin)	0.25	3	5.75
Solvent-to-sample ratio	10	30	50
Extraction time	30	60	90

4. Conclusions and future perspectives

The aim of this PhD project is the optimization of a green extraction technique using Natural HDESs for the recovery of carotenoids from by-products of the industrial processing of vegetable foods, as well as from an unconventional food source, the microalga *Chlorella vulgaris*. The initial activities were carried out using the scientific approach of carefully reviewing the pertinent literature in the field of DESs, to identify a promising research gap to work on.

Nowadays, the use of Natural HDESs as solvents for the extraction of apolar molecules from food matrices has been investigated only in a limited number of papers, while the expansion of the knowledge in this area is of noticeable interest. The research involved the study of the physicochemical characteristics of 18 Natural HDESs as well as their use at preset conditions to identify the most suitable ones for the carotenoid extraction. Out the 18 solvents, 3 were selected for further investigation on real industrial substrates and 2 for the microalgae. The extraction process was studied and optimized for the vegetable by-products, while for microalgae this part of the research is undergoing, ending this PhD project in March 2024. In the meantime, recovery and recycling of the Natural HDESs will be investigated, as well as the possibility to incorporate the extracts in food supplements and/or cosmetic products, carrying out the study in cooperation with companies of the field, which have shown interest in the research.

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Innovative approach to design cereal-based product with low glycemic response

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The aim of this project is to investigate, from micro to macro, the effect of structural features, such as cell wall intactness, protein matrix, and food texture and their interactions, on the starch digestibility of bread. In the first two chapters of the Ph.D. program, the role of the cell wall integrity and textural features on the starch digestibility of durum wheat and rye flour and bread produced with these flours was studied. From each cereal, three particle sizes were produced, i.e., small (<350 µm), medium (1000 µm-1800 µm), and large (> 1800 µm) flour. For both cereals, the presence of a cluster of intact cell wall decreased the starch digestibility in flour acting as a barrier between starch and enzyme, but this effect of protection was lost with bread production. It was hypothesized that the long mixing time of medium and large flours needed to reach an optimum developed dough increased the cell wall porosity due to the solubilization of their components and in turn the enzyme penetration. Moreover, the use of coarse flour reduced the cohesiveness of the bread crumb, increasing the disintegration rate during digestion and, in turn, the starch accessibility. Based on these results, in the third chapter of the thesis, it was evaluated the effect of reduced mixing time and increased cohesiveness of bread crumb on textural features and starch digestibility of bread made with coarse durum wheat (>1000 µm). To increase the bread cohesiveness, two approaches were evaluated: the decrease in bread moisture content and the substitution of 20% of coarse semolina with vital gluten. The final aim was to identify the best recipe to obtain bread with reduced accessibility and acceptable textural quality to be tested in an acute study in humans.

Approccio innovativo nella progettazione di prodotti da forno a bassa risposta glicemica

Lo scopo di questo progetto è quello di indagare, dal micro al macro, l'effetto di diverse caratteristiche strutturali, come la presenza della parete cellulare intatta, la matrice proteica, la texture e le loro interazioni, sulla digeribilità dell'amido del pane. Nella prima parte del dottorato è stato studiato il ruolo dell'integrità della parete cellulare e delle caratteristiche strutturali sulla digeribilità dell'amido della farina di grano duro e di segale e del pane prodotto con le stesse farine. Da ciascun cereale sono state prodotte tre granulometrie, ovvero fine (<350 µm), media (1000 µm-1800 µm) e grossolana (> 1800 µm). Per entrambi i cereali, la presenza di cluster di cellule integre diminuisce la digeribilità dell'amido nella farina, agendo da barriera tra l'amido e l'enzima; tuttavia, questo effetto di protezione viene perso quando da queste farine vengono prodotti i pani. Si è ipotizzato che durante la lunga miscelazione (fino a 90 min per la farina grossolana) le pareti cellulari aumentano la loro porosità a causa della solubilizzazione dei componenti delle pareti cellulari aumentando la diffusività degli enzimi all'interno della cellula. Inoltre, l'uso di semola grossolana può aver ridotto la coesività della mollica, aumentandone la velocità di disgregazione durante la digestione e, a sua volta, l'accessibilità all'amido. Sulla base di questi risultati, nel terzo capitolo della tesi, è stato valutato l'effetto della riduzione del tempo di miscelazione e dell'aumento della coesività della mollica sulla texture e sulla digeribilità dell'amido di pani prodotti con semola grossolana (>1000 µm). Al fine di modificare la consistenza del pane prodotto con la semola grossolana sono stati utilizzati due approcci, quello di ridurre l'idratazione dell'impasto e la sostituzione del 20% di semola con glutine vitale. Lo scopo finale è stato quello di individuare, tra i campioni testati, il pane con minore digeribilità dell'amido ma accettabili caratteristiche strutturale, da testare in uno studio in acuto nell'uomo.

Keywords: *durum* wheat; rye; gluten; cohesiveness; hardness; *in vitro* starch digestion.

1. Introduction

Worldwide, the number of people suffering from type 2 diabetes is around 422 million and this number is continuously rising (World Health Organization, 2021). The spread of diabetes in the last decades is the result of a global rise in obesity, a more sedentary lifestyle, and an energy-dense diet, given by the overconsuming of mainly highly digestible starchy food (Chatterjee et al., 2017). Among highly digestible starchy foods, bread is a staple food daily consumed in Western countries and is characterized by a high glycemic index (GI). For this reason, how decreasing the blood glucose response of starchy food, such as bread, and consequentially its GI has been extensively studied in the last decades. Limiting starch accessibility to α-amylase is a promising approach to decrease starch accessibility (Rovalino-Córdova et al., 2019). In plant food, starch granules are naturally encapsulated in the cell. In cereals, the intact cells could limit the accessibility to starch when isolate cells, flours

(wheat, sorghum, and barley) and simple food product, such as porridge, are studied both *in vitro* and *in vivo* (Bhattarai et al., 2018; Edwards et al., 2015; Korompokis et al., 2019), However, contradictory results were found when the effectiveness of coarse flour with large particle size rich in clusters of intact cells was investigated in bread. Lin et al., (2020) found that increasing particle size of whole wheat flour significantly decreased starch digestibility in bread, whereas (Korompokis et al., 2021) demonstrated that the incorporation of coarse flour did not have an effect in modulating the rate of starch digestibility in bread. In bread, not only the cell wall can act as a barrier limiting the contact between starch and enzyme but also protein, the second macronutrient present in cereals, also has a role in this sense. Gliadin and glutenin, which are the main protein of some grains, after hydration and mixing force, form a discontinuous network that surround the starch granules which could decrease the digestibility limiting the starch accessibility (Chen et al., 2019). Gluten, moreover, not only can physically hamper the contact between starch and enzyme, but it was demonstrated that this protein complex could bind the pancreatic alpha-amylase, and consequentially inhibit starch digestibility (López-Barón et al., 2017). In the same direction, the food structure also plays an important role in the digestion and absorption of nutrients. Bread texture could affect bread mastication and consequentially the bolus disintegration during the gastric phase. It was proven that the relatively big size of compact digesta could limit the diffusivity of the enzymes inside the bread structure, delaying and limiting the starch digestibility (Martínez et al., 2018). **The aim** of this project is to investigate, from micro (cell wall intactness and protein matrix) to macro (food texture), the effect of structural features on the starch digestibility of bread. In accordance with the Ph.D. thesis project previously described (Tagliasco, 2021), this oral communication reports the main results of the following three research activities: A1) Monitoring the effect of cell wall integrity in modulating the starch digestibility of durum wheat during different steps of bread making. A2) Role of particle size in modulating starch digestibility and textural properties in rye flour and bread model system. A3) The effect of gluten addition, dough moisture content, and different mixing time, on the textural properties and *in vitro* starch digestibility of durum wheat bread made with coarse semolina.

2. Experimental plan

A1) Monitoring the effect of cell wall integrity in modulating the starch digestibility of durum wheat during different steps of bread making. (Tagliasco et al., 2022)

The aim of the first study was to evaluate the effect of three semolina particle sizes, i.e., small (<350 µm), medium (> 1000 µm < 1800 µm), and large (> 1800 µm), on starch digestibility in raw semolina, dough, and bread, to better understand how the processing affects the ability of cereal cell wall to act as a barrier to the enzyme accessibility. The aim was to determine at which stage of the baking process the physical encapsulation of starch within cell walls lost its ability to effectively reduce the starch *in vitro* digestibility.

Materials and methods

Peeled durum wheat grain was purchased from Duru BakliyatTM (Hediklik Dis¸ Bug˘dayı, Turkey). Small (<350 µm), medium (> 1000 µm < 1800 µm), and large (> 1800 µm) particle size flours were obtained with a pin mill (Multi-mill, Alpine Hosokawa, Augsburg, Germany). The dough was prepared according to a standard recipe with 1.2% yeast and 1% salt as % wet flour basis and optimized, in terms of moisture content and mixing time, to obtain dough with the same consistency (500 Brabender Units). Small flour needed 5 min of mixing, medium one 60 min and large one 90 min. The starch digestibility of flour, dough, and bread was tested with the in *vitro* Englyst's method (Englyst et al., 1992). Confocal laser scanning microscopy was used to check the presence of intact cells in each step of baking. Texture analyses were conducted to evaluate the textural features of bread made with increasing flour particle size.

Results and discussion

The images from the confocal laser scanning microscopy showed that the integrity of the cell wall (stained in blue) was kept during the whole bread processing for the medium and large particle size flours (MF, LF, MD, LD, MB and LB) whereas cell walls were mostly destroyed in the flour of small particle size (SF, SD and SB). *In vitro* starch digestibility of flour decreased, increasing particle size. This effect can be mainly ascribed to the presence of a higher fraction of intact cells, which acts as a barrier limiting the contact between enzyme and starch, in the flour of medium and

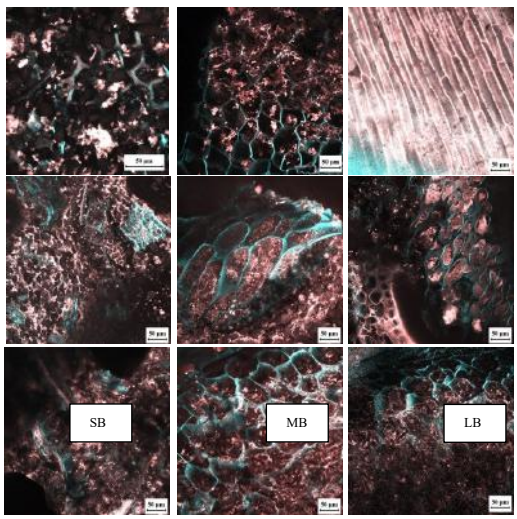

Figure 1. Confocal laser scanning microscopy images of small flour (SF); medium flour (MF); large flour (LF); small dough (SD); medium dough (MD); large dough (LD); small bread (SB); medium bread (MB); large bread (LB).

large size than in fine flour. For what concern the dough, no difference in starch digestibility was found as shown in Table 1. This indicates that the effect of large particle size was no longer able to modulate the starch digestibility even though intact cells were still present in the middle and large particle size dough. Therefore, we hypothesize that, during the long mixing time (60 and 90 min, respectively for the medium and large flour) and fermentation steps, the porosity of the cell walls increased due to the solubilization of the main components of the wheat cell wall. For what concern bread, instead, a modest decrease in starch digestibility for bread made by large particles was observed, likely due to its dense structure. Bread made with large particle size flour, indeed, was more compact and denser than those made with medium and small flours. This difference in the texture could have delayed the lower rate of starch digestion than other bread types.

Table 1. Rapidly digestible starch (RDS); slowly digestible starch (SDS) and resistant starch (RS) of flour, dough, and bread made with small flour (< 350 µm), medium flour (> 1000 µm; < 1800 µm) and large flour (> 1800 µm).

		RDS (g/100 g total starch)	SDS (g/100 g total starch)	RS (g/100 g total starch)
Flour	Small	30.4 ± 4.2^{a}	63.4 ± 2.6^{a}	6.8 ± 1.1^{c}
	Medium	16.5 ± 3.2^{b}	60.4 ± 5.6^{a}	25.6 ± 3.9^{b}
	Large	8.9 ± 0.1^{c}	36.0 ± 1.8^{b}	56.1 ± 2.4^{a}
Dough	Small	32.0 ± 4.5^{A}	42.3 ± 15.3^{A}	26.3 ± 12.9^{A}
	Medium	25.9 ± 9.3^{A}	48.5 ± 13.2^{A}	25.6 ± 13.6^{A}
	Large	25.8 ± 6.4^{A}	48.9 ± 4.2^{A}	28.5 ± 8.1^{A}
Bread	Small	68.4 ± 9.7^{a}	22.5 ± 6.4^{b}	7.6 ± 1.6^{a}
	Medium	59.1 ± 14.1^{ab}	33.7 ± 4.6^{a}	9.2 ± 0.2^{a}
	Large	55.7 ± 5.8^{b}	37.2 ± 3.5^{a}	3.7 ± 0.7^{b}

The same letter indicates no significant difference among the three particle sizes for flour, dough, and bread for each column ($p < 0.05$, Tukey's test, n =3).

A2) Role of particle size in modulating starch digestibility and textural properties in a rye bread model system. The second study elucidates the effect of clusters of intact cells on the starch digestibility of rye flour and a model rye bread. The textural quality, *in vitro* starch digestion, and physical disintegration during the digestion were investigated to study the relationship among the integrity of cell walls, the structural features of bread, and the *in vitro* starch digestibility.

Materials and method

Rye (*Secale cereale L.*) grain was purchased from Tibiona (Villanova Mondovi, Italy) and ground using a multi mill (Alpine Hosokawa, Augsburg, Germany) to obtain three particle size flours: small rye (S) (<350 µm), medium rye (M) (> 1000 µm < 1800 µm), and large rye (L) (> 1800 µm). the bread was prepared following a standard recipe with 2% yeast and 1% salt as % wet flour basis. The starch digestibility of flour and bread was tested with the in *vitro* Englyst's method (Englyst et al., 1992). Texture profile analyses (TPA) was conducted to evaluate the textural features of bread. The disintegration of the samples during the *in vitro* digestion was studied by image analysis, measuring the particle size of the digesta over time.

Results and discussion

In the present study, the effect of three different particle sizes on the digestibility and textural quality of rye flour and bread produced therefrom were investigated. The starch digestibility of small rye flour was higher than medium and large ones. In bread, instead, the results obtained were quite unexpected. Rapidly digestible starch (RDS) (Table 2) was not significantly different among the three bread samples. Instead, slowly digestible starch (SDS) was significantly lower in bread made with small particles (SB) than the ones made with medium (MB) and large particles (LB). Probably during bread processing the cell wall was not damaged, but its porosity increased due to the solubilization of arabinoxylans. Moreover, the differences found for SDS and resistant starch (RS) among the three samples could be also ascribed to the distinct texture of bread. As shown in Figure 2, the crumb cohesiveness, which represents the ability of the crumb to regain its height after a stress, decreased drastically with the increase in particle size. The texture parameters mirrored the different disintegration behavior observed during the *in vitro* digestibility. Indeed, MB and LB, which were characterized by a lower cohesiveness compared to SB, produced more small particles after the first 20 min of digestion (data not shown). The relatively bigger size of the compact digesta particles in SB could instead have limited the diffusivity of the enzymes inside the bread structure, slowing down starch digestibility, and therefore produced a higher amount of starch that escaped digestion, i.e., RS. In conclusion, the intactness of cell walls is a limiting factor that controls the extent of hydrolysis of starch only in rye flour but not in a bread matrix. Instead, bread that disintegrated less

Table 2. Rapid digestible starch (RDS); slowly digestible starch (SDS) and resistant starch (RS) of flour, dough and bread made with small, medium and large rye flour and bread.

		RDS (g/100 g total starch)	SDS (g/100 g total starch)	RS (g/100 g total starch)
Flour	Small	26.1 ± 4.1^{a}	71.8 ± 7.4^{a}	2.1 ± 1.4^{c}
	Medium	18.2 ± 2.7^{b}	60.3 ± 7.2^{a}	23.1 ± 7.7^{b}
	Large	14.1 ± 0.1^{b}	55.5 ± 0.3^{b}	30.1 ± 1.7^{a}
Bread	Small	56.2 ± 7.4^{a}	32.1 ± 3.4^{b}	26.1 ± 10.4^{a}
	Medium	49.3 ± 3.6^{b}	59.4 ± 9.3^{a}	5.5 ± 4.9^{b}
	Large	58.6 ± 11.1^{a}	48.2 ± 4.5^{a}	6.1 ± 5.8^{b}

during digestion was the one with the lower starch accessibility.

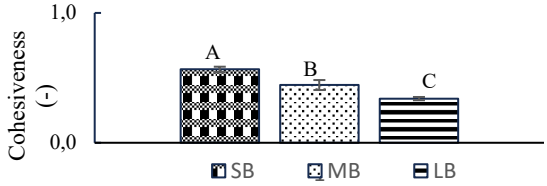

Figure 2. Cohesiveness (-) of rye bread made with small (SB), medium (MB) and large (LB) particle flours. Columns sharing the same letter were not significantly different (p<0.05, Tukey's test, n =9).

*The same letter in the same column indicates no significant differences ($p < 0.05$, Tukey's test, n =3).

A3) The effects of gluten addition, dough moisture content, and different mixing time, on the textural properties and *in vitro* starch digestibility of durum wheat bread made with coarse semolina.

The third study aims to elucidate the effect of dough mixing time and different textural characteristics on the starch digestibility of wheat durum bread prepared with coarse semolina (particle size > 1000 µm). To change the crumb texture, two approaches were evaluated: the decrease in bread moisture content and the substitution of 20% of coarse semolina with vital gluten.

Materials and methods

Peeled durum wheat grain was purchased from Duru BakliyatTM (Hediklik Dis ̧ Bug ̆dayı, Turkey) and milled with a pin mill (Multi-mill, Alpine Hosokawa, Augsburg, Germany) to obtain particle size > 1000 µm. Six durum wheat bread samples were prepared using only coarse semolina (S, particle size > 1000 µm) or 20% vital gluten Primeal (Peaugres, France) in substitution of S, 70% of water (optimum water absorption) or 55% (low water absorption) and different mixing times 5 min (short mixing time) and 45 min (optimum mixing time to obtain a dough with 500 BU). Textural properties were evaluated by a texture profile analysis (TPA) and *in vitro* starch digestibility was assessed according to Englyst's method.

Results and discussion

The results of the study showed that the gluten-enriched bread samples exhibited, in general, better textural properties: lower hardness, higher cohesiveness, and bigger volume, than the samples produced with only coarse semolina (Table 3). Only, the sample, 80Semolina+20Gluten_5min_70%moisture behaved differently, having the same volume and hardness as the bread sample 100Ssemolina_45min_70%moisture. This could be due to the high hydration level and the short mixing time; 5 minutes of mixing are probably not enough to absorb all the water added to the flour and, as a result, the bread structure collapsed. For what concern the starch digestibility bread made only with semolina at low hydration (100S_45_55%) had significantly lower RDS than bread made with 20% of gluten and optimum mixing time and hydration level (80S+20G_45min_70%). Moreover, the sample 100S_45_55% showed the smallest volume and hardest crumb texture, instead, 80S+20G_45min_70% had the least hardness and biggest volume. It is clear from these results that during the first 20 min of digestion, the starch is more accessible, and easily digested, due to the aerated crumb structure. This is also confirmed by the significant positive correlation (r= 0.854) between volume and RDS, the higher the bread volume, the higher is RDS value. However, during the further 100 min of digestion, the trend changes, the samples with the highest cohesiveness turned out to be the least digestible. This is probably due to the cohesive structure of the bread that during digestion which could limit the crumb disintegration and consequently starch accessibility. SDS indeed is highly negatively correlated with the cohesiveness results (r=-0,905). These results demonstrated the pivotal role of textural characteristics on the starch digestibility of bread.

Table 3. Volume (cm^3), hardness (N), cohesiveness (-), rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) of coarse semolina bread samples

	Volume (cm^3)	Hardness (N)	Cohesiveness (-)	RDS (g/100 g total starch)	SDS (g/100 g total starch)	RS (g/100 g total starch)
80S+20G_5min_70%	105.0 ± 5.0^{c}	22.3 ± 3.2^{b}	0.76 ± 0.01^{b}	48.1 ± 2.5^{ab}	33.9 ± 2.0^{b}	18.0 ± 3.3^{a}
80S+20G_5min_55%	205.0 ± 8.6^{b}	4.5 ± 0.7^{c}	0.81 ± 0.01^{a}	46.5 ± 2.1^{ab}	35.0 ± 2.9^{b}	18.5 ± 4.6^{a}
80S+20G_45min_70%	315.0 ± 13.2^{a}	3.8 ± 0.4^{c}	0.82 ± 0.01^{a}	54.4 ± 3.0^{a}	33.9 ± 4.7^{b}	11.6 ± 7.8^{a}
80S+20G_45min_55%	286.7 ± 31.7^{a}	4.0 ± 0.7^{c}	0.81 ± 0.02^{a}	50.0 ± 4.7^{ab}	30.8 ± 2.8^{b}	19.1 ± 2.3^{a}
100S_45_70%	111.7 ± 12.6^{c}	23.8 ± 0.0^{b}	0.71 ± 0.01^{c}	46.8 ± 1.5^{ab}	44.0 ± 10.7^{ab}	9.1 ± 10.4^{a}
100S_45_55%	90.0 ± 13.2^{c}	43.8 ± 10.5^{a}	0.70 ± 0.01^{c}	42.9 ± 3.6^{b}	49.8 ± 7.3^{b}	7.3 ± 4.6^{a}

S; semolina, G; gluten, 5 and 45 min; mixing time, 55% or 70%; moisture absorption. The same letter in the same column indicates no significant differences ($p < 0.05$, Tukey's test, n =3)

3. Conclusions and Future Perspectives

In conclusion, as shown from the PCA (Figure 3), the bread sample produced with the addition of 20% gluten, low hydration (55%) level, and 5 min of mixing, was the best compromise between acceptable textural features such as high cohesiveness and low hardness, and low starch digestibility. The latter was well correlated with RS and inversely correlated with SDS. This could be explained by the preservation of cells' wall integrity, associated with the effect of the gluten network that was able to hamper the enzyme and the presence of a cohesive crumb texture which didn't disintegrate during digestion. However, these results must be confirmed by a human study in which the effect of these bread characteristics is evaluated on oral processing and the consequent effect on glucose release. For this reason, the next study will aim to evaluate the glycemic and insulinemic response in healthy volunteers of bread made with durum wheat coarse flour and gluten compared to a standard durum wheat bread made with fine semolina. Moreover, the oral processing of bread samples will be studied to evaluate the effect of gluten on oral disintegration, inhibition of salivary alpha-amylase, and consequentially glucose release.

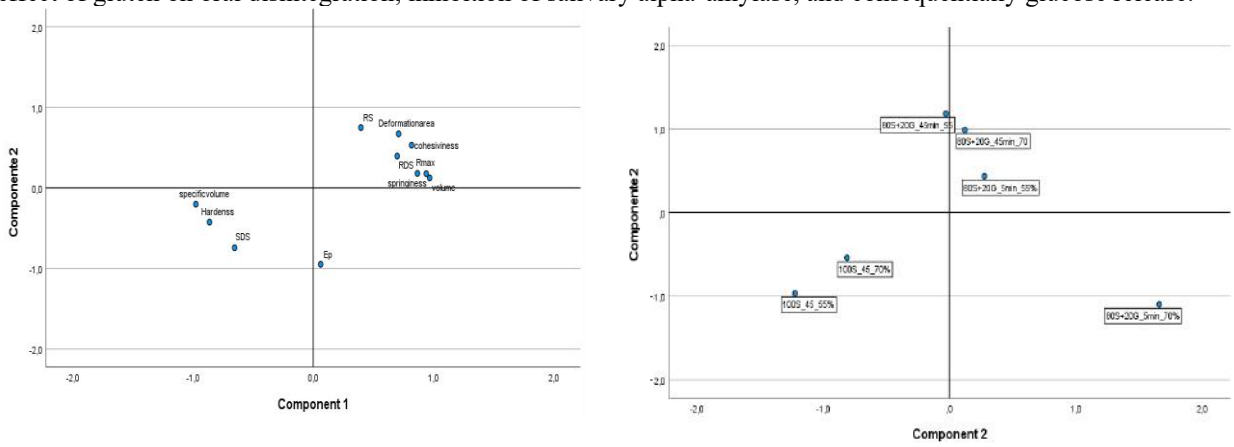

Figure 3. Data of bread characteristics displayed through the first two principal components (PC1 and PC2) derived from the PCA (principal components analysis) (A): biplot of the first two components; (B): rotated principal scores of bread samples produced. volume; hardness; springiness; cohesiveness; deformation area, extensibility peak (Ep), the maximum force of extensibility (Rmax); rapidly digestible starch (RDS); slowly digestible starch (SDS); resistant starch (RS).

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Fusarium musae, a potential new food safety threat. Can a diseased banana be the source of a fungal disease for humans?

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This PhD thesis dealt with the assessment of a comprehensive analysis of a cross-kingdom pathogen such as *Fusarium musae* by combining studies within different disciplinary field. This research project aims at characterising *F. musae* to document the diversity of the species, to better understand its mechanisms of infection and its ability to be transmitted from banana to humans.

Fusarium musae, una nuova potenziale minaccia per la sicurezza alimentare. Può una banana malata essere fonte di malattia fungina per l'uomo?

Questa tesi di dottorato riguarda lo sviluppo di un'analisi completa di un patogeno cross-kingdom come *Fusarium musae* combinando conoscenze in campi disciplinari diversi. Questo progetto di ricerca mira a caratterizzare *F. musae* documentando la diversità della specie, ad avere una visione più completa sui suoi meccanismi di infezione e la capacità di essere trasmessa da banana a uomo.

Key words: fungal characterization; infection mechanisms; fungal transformation; *Fusarium musae*.

1. Introduction

In accordance with the PhD thesis project previously described (Tava, 2021), this oral communication reports the main results of the following four activities directed to:

A1) characterise *F. musae* by testing its sensitivity to different azoles used in clinical and agricultural fields. We collected 18 strains of *F. musae* isolated worldwide from both banana fruits and human patients, eight DMIs used in crop protection and five medical antifungals were tested against the entire collection;

A2) genomics analysis of the entire collection of *F. musae* strains to study fungal diversity in relation to geographical and host origin. I obtained the first genome of *F. musae* complete at chromosomal level, whole genome of reference strains isolated one from banana fruits and one from human infection were analysed and compared. Mitogenomes of the entire population were assembled and comparatively analysed;

A3) proof experimentally for the first time the ability of *F. musae* to cause infection in both animal and plant kingdoms by building two infection models for banana fruits and *Galleria mellonella* (chose as "human proxy") that could be representative of the infection;

A4) construction of fluorescent reporter strains to be used for host-pathogen interaction studies. Representative strains of *F. musae* were transformed and fluorescent strains were obtained for *in vivo* imaging of the infection in the two infection models established as described in A3.

2. Material and Methods

(A1) All molecules were prepared at final concentrations ranging from 0,03 to 16 mg/L according to the Clinical and Laboratory Standards Institute guidelines for filamentous fungi (Reference CLSI M38-A2). Inoculum suspensions were prepared from 2–5-day-old cultures diluting to a final working inoculum of 0,5–5 × 10^4 CFU/mL. Plates were incubated at 28°C for 48 h. The minimum inhibitory concentration (MIC) value was the concentration of drug yielding no fungal growth at visual reading. Tests were performed in duplicate.

(A2) DNA used for sequencing was obtained from fresh mycelia of 17 strains according to a modified CTAB method (Pasquali *et al.*, 2004), followed by Genomic tips column purification (Qiagen, Germantown, MD, USA). Sequencing was carried out using Illumina Hiseq 2000 (151 bp x2) by Novogene (Cambridge, UK). Mitogenomes of NRRL25059 strain was obtained from the NCBI database. Assembly was carried out de novo with NOVOplasty 4.2. Mitogenomes were then annotated by integrating MFannot and RNAWeasel. Sequences were then aligned using the MAFFT alignment tool using Geneious prime software and manually checked and analyzed using Median Joining Network in PopArt (Degradi *et al.*, 2022).

Complete genome of one representative strain was obtained by combining Illumina with long-read sequencing

using the MinION MIN101B platform, R9.4.1 flow cell (Nanopore). Assembly was performed using Canu v.2.1.1 + galaxy0. Autopolishing was performed using Medaka v.1.0.3 + galaxy2. Minimap2 v.2.17 + galaxy4 was used to align short reads on the obtained assembly. Manual correction was done using Geneious Prime software v.11 (Biomatters) and final assembly statistics were evaluated using Quast tool v.5.0.2 + galaxy1 (Degradi *et al.*, 2021).

(A3) For infection of both banana fruits and *G. mellonella,* strains were grown in CMC liquid medium, spores were harvested after 5 days by filtering through one-fold miracloth and counted with Burker count chamber. Appropriate dilutions were made to obtain the required concentration of 10^5 spores/ml.

Bananas were sterilized by immersion in bleach 0,7% for 3 min, wash in deionized sterile water for few seconds and dry under sterile laminar flow hood on a sheet of paper. In the meanwhile, 20 sterile toothpicks were immersed in each conidia suspension for few minutes. Five toothpick per fruit were positioned at a regular distance deep enough to break the peal and touch the surface of the pulp. Groups of 4 bananas fruits were used for each strain, additional control group of 4 bananas inoculated with sterile water was also included. After infection I placed bananas on trays, covered by plastic bags and incubated at 20°C in the dark for 10 days. Infections were performed in three separate occasions.

Levels of infection were estimated by measuring the diameter of the spots grown on the banana fruits and a number from 0 to 4 was assigned to each spot based on halo, browning and mycelium formation. To compare the virulence of the different strains I calculated an overall index that could be comprehensive of both two parameters: $\frac{(disease\ scale)^2 + average\ diameter}{2}$. Overall indexes calculated were plotted with the data analysis framework Estimation Stats with default parameters.

Specimens of *Galleria mellonella* at the larval final stage were bought from Biosystems Technology Ltd (Tanners' Yard, 100 High Street, Crediton, Devon, EX17 3LF) and maintained at 16°C until the time of infection within 7 days of arrival at our laboratory. I selected groups of 10 larvae confirming they were completely healthy and infected them with 10µL of each inoculum suspension with a 1ml insulin syringe into the last left proleg of the larva. Additional control group of 10 larvae injected with 10µL of PBS was also included. Larvae were incubated at 28°C for up to 7 day, twice a day each group of larvae was observed and each larva was awarded a score based on the level of activity, pigmentation, cocoon formation and state of life following the values shown in Table 1.

Levels of infection were estimated by calculating the sum of scores obtained for each larva. To compare the virulence of the strains of our collection we calculated an overall index comprehensive of these parameters: $\frac{[9-(sum*survival)]}{10}$. Death larvae at each check point were removed and placed at -20°C for at least 48h before being disposed of. Overall indexes calculated were plotted with the data analysis framework Estimation Stats with default parameters. Survival curve was plotted with the Kaplan-Meier method using SPSS Statistics (version 27).

CATEGORY	DESCRIPTION	SCORE
activity	no activity	0
	minimal activity on stimulation	1
	active when stimulated	2
	active without stimulation	3
melanization	complete melanization	0
	dark spots on brown wax worm	1
	>3 spots on brown wax worm	2
	<3 spots on brown wax worm	3
	no melanization	4
cocoon formation	full	0
	partial	0,5
	no	1
survival	dead	0
	alive	1
	total	sum

Table 1 *Representation of the categories and scores used to assess the level of disease caused by each strain in G. mellonella.*

(A4) Two representative strains of *F. musae* were chosen to obtain fluorescent reporter strains, one isolated from banana fruits and one isolated from human patient. Genomic data allowed the construction of a specific plasmid containing the far-red fluorescent protein E2-Crimson expressed under control of the constitutively active *F. musae* enolase promoter *Peno1.* For transformation, we firstly obtained Competent cells from single colonies of *E. coli* DH5α by heat-shock protocol and then we transformed them with E2-Crimson plasmid following Thermoscientific user guide. Selected single colonies were grown in liquid media for plasmid DNA extraction using QIAGEN Plasmid Midi Kit following manufacture's protocol. For fungal transformation protoplasts of the two selected strains were obtained and transformed with linearized plasmid DNA as described in (Liu and Friesen, 2012).

Success of transformation was confirmed with confocal fluorescence microscope (Nikon A1-3D SIM) using TRITC filter.

3. Results and Discussion

3.1 Azoles sensitivity

Each azole has a unique spectrum and power as represented by different MIC values in Figure 1. Results showed that itraconazole presented highest MICs (G-MIC = 16 mg/L) among the medical azoles against *F. musae*, while posaconazole, voriconazole and isavuconazole showed lower values (respectively 0.54 mg/L; 1.2 mg/L and 2.4

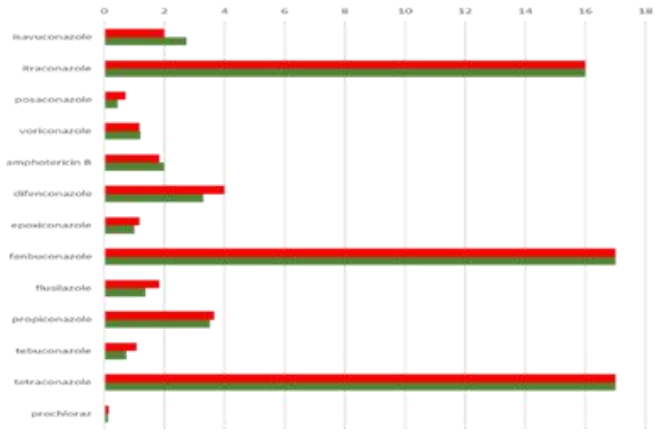
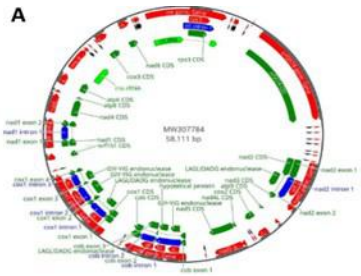


Figure 1 *Susceptibility to antifungal drugs of F. musae isolates from human (upper line) and bananas (lower line).*

mg/L); amphotericin B showed a G-MIC of 1.93 mg/L. Prochloraz and the tebuconazole (G-MIC of 0.14 mg/L and 0.83 mg/L respectively) presented the highest activity among agricultural azoles, while higher values were showed by difenoconazole (2–8 mg/L), propiconazole (2–4 mg/L) and flusilazole (1.55 mg/L). Tetraconazole and fenbuconazole showed G-MICs > 16 mg/L for all strains. Overall our data resulted comparable with data already present in literature and we did not observed statistically significant differences linked to the different geographical and host origin of the strains of our collection (Tava *et al.*, 2021).

3.2 Mitogenomes

Mitogenomes of *F. musae* strains ranged from 56,439 to 59,256 bp (Figure 2A). The analysis of codon usage in coding sequences did not reveal significant differences among the whole set of analyzed strains, all mitogenomes showed a high similarity for protein-coding regions. However, our study confirmed that intergenic regions and endonucleases may be exploited to identify subgroups within a species. Different nuclear gene haplotypes exist and at least one set of strains belonging to the same mitochondrial haplotypes included both human and banana derived strains (Figure 2B) suggesting that the species can travel as hypothesized by (Triest and Hendrickx,



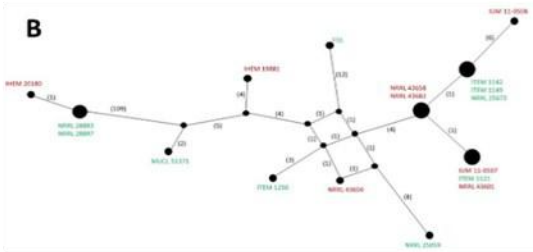


Figure 2 *(A) Graphical representation of circular mtDNA of F. musae. (B) Representation of mitogenomes network haplotypes using PopArt software.*

2016).

3.3 Genome

Strain F31 presented a size of 44.07Mb divided into 12 chromosomes of which 11 have both telomers, the circular mitochondrial DNA (mtDNA), and one unplaced contig. Functional annotation led to a total of 13,963 annotated genes, of which 13,661 were proteins and 302 transfer RNA (tRNA) for nuclear DNA. This represents the first genome completed at chromosome level of *F. musae* and it will be a useful resource for comparative analysis of *F. musae* species, in addiction it represents an important reference for completeness and for understanding the genome evolution in the FFSC.

3.4 Banana fruits infection

Results of infection of bananas demonstrated for the first time that human strains of *F. musae* are able to invade a plant host. All banana fruits infected with *F. musae* strains presented brown spots surrounding the point of toothpick insertion, symptoms varied from small halo with browning to big brown spot with spread visible

mycelium proving that *F. musae* can be pathogenic of banana fruits. All strains presented level of infection

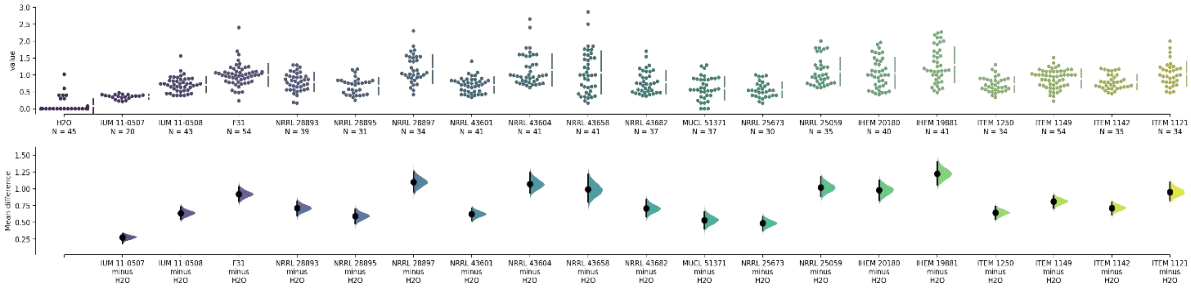


Figure 3 *Distribution of overall indexes calculated for each infection point in banana fruits. Graph was made with the data analysis framework Estimation Stats with default parameters, H_2O was used as shared control. Each mean difference is depicted as a dot and each 95% confidence interval is indicated by the ends of the vertical error bars. All data are normalised by dividing for average value of F31 of the corresponding replicate.*

statistically different from H2O, as represented in Figure 3, demonstrating that they were all able to cause significant disease in banana fruits. Geographical origin and host of original isolation did not correlate with the severity of the disease, all strains cause comparable disease level and no subgroup are present in our population based on their origin.

3.5 *Galleria mellonella* infection

Galleria mellonella assay showed that *F. musae* causes visible disease in *G. mellonella* that in this way can be considered as a valid "human proxy" model for the investigation of this novel fungal species. Of the nineteen *F. musae* strains tested, five (three human IUM 11-0507, NRRL 43601, NRRL 43604 and two plant NRRL 28893, ITEM 1149) were not statistically different from PBS. Overall, all *F. musae* strains caused comparable levels of infection in *G. mellonella* (Figure 4) and no subgroups were present based on isolation source.

Additional Survival analysis was performed on the entire collection of *F. musae* strains. Inoculation of *G. mellonella* with all nineteen *F. musae* resulted in significant killing of the larvae, 96h post infection we started seeing a conspicuous number of death larvae that increased the days after. This demonstrated that also strains isolated from plants are potentially capable to cause infection in animal pathosystem.

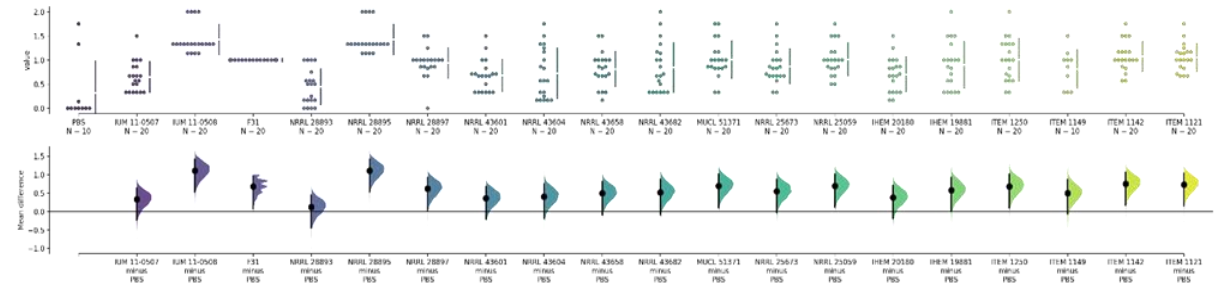


Figure 4 *Distribution of overall indexes calculated for each infection point in G. mellonella. Graph was made with the data analysis framework Estimation Stats with default parameters, PBS is used as shared control. Each mean difference is depicted as a dot and each 95% confidence interval is indicated by the ends of the vertical error bars. All data are normalised by dividing for average value of F31 of the corresponding replicate.*

3.6 Fluorescent reporter strain

Fungal transformation resulted in the production of two fluorescent representative *F. musae* strains that open possibilities for the investigation *in vivo* of the interaction between *F. musae* and its hosts. One banana strain and one human strain were successfully transformed as we could verify by visualization with confocal fluorescence microscope (Figure 5).

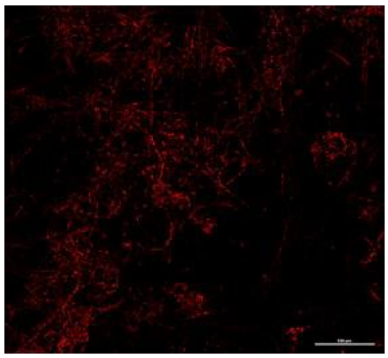


Figure 5 *Representative picture of F. musae transformed with E2-Crimson observed with confocal fluorescence microscope (Nikon A1-3D SIM) using TRITC filter. From the picture we can clearly distinguish hyphae of the colony.*

4. Conclusions and Future Perspectives

The "One Health" concept is based on the idea that the health of people is closely connected to the health of animals and our shared environment. Food safety has a crucial role within the "One Health" concept given the emerging threat pose by some fungal pathogens of food crops that can mine also human health. In recent years, the number of fungal pathogenic species able to cross kingdom borders is increasing, becoming of interest of both clinical and agricultural field (van Baarlen *et al.*, 2007; Kim *et al.*, 2020). This implies that plants (and food products derived) can become an important source of novel infecting agents for humans, and studying plant pathogens can contribute to establishing novel standards of food safety which will have to consider also the risk caused by this new category of pathogens. Very limited information is available for fungal agents and their mechanisms of action and in most of the cases their ability to "jump" from one host to another is not experimentally verified following Koch's postulate.

With my PhD thesis I explored the potential cross-kingdom pathogenicity of *Fusarium musae*, recently isolated from banana fruits (Van Hove *et al.*, 2011; Kamel, Cortesi and Saracchi, 2016) and human patients (Esposto, Prigitano and Tortorano, 2016; Triest *et al.*, 2016). My project aimed at characterising this novel species by combining studies within different disciplinary field to understand peculiarities of the species as well as to explore a successful strategy that could be used as model for the investigation of other cross-kingdom pathogens. Fungicide sensitivity and genome sequencing highlight important characteristics for a correct identification of the *F. musae*. Infection assays prove experimentally for the first time the ability of *F. musae* to cause evident disease in a plant pathosystem as well as in an animal pathosystem confirming *F. musae* as a potential cross-kingdom pathogen.

Florescent strains will be a useful tool for future investigation *in vivo* of the interaction between *F. musae* and its hosts, and to better understand it's mechanisms of action.

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Advancement and prospects of study of bioactive peptides during food fermentation

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In the current trend favoring plant-based foods over animal-based foods, pulses offer an alternative source of protein as well as bioactive peptides (BPs). We examined how proteins in a red lentils protein isolate (RLPI) break down during fermentation with different lactic acid bacteria and yeast strains. *Hanseniaspora uvarum* SY1 and *Fructilactobacillus sanfranciscensis* E10 were the most effective microorganisms in terms of protein hydrolysis. *H.uvarum* SY1 produced the highest levels of antiradical, ACE-inhibitory, and antifungal activities in the low molecular weight water soluble extracts (LMW-WSE). We analyzed the 2039 peptide sequences identified in the LMW-WSE using the BIOPEP UWM database, and 36 sequences matched with known BPs. Fermentation generated 12 peptides that were not present in raw RLPI. Furthermore, *H.uvarum* SY1 resulted in the highest quantities of BPs, particularly those with antioxidant and ACE-inhibitory properties. The peptides KVI, LVR, and LVL were identified for the first time in the fermented samples. Additionally, we found 44 new potential BPs that exhibited antifungal activity and are deserving of further investigation and characterization.

Avanzamento e prospettive di studio dei peptidi bioattivi durante la fermentazione di matrici alimentari

Nella tendenza attuale che vede i cibi di origine vegetale preferiti rispetto a quelli di origine animale, i legumi offrono un'alternativa come fonte di proteine e di peptidi bioattivi (BPs). Abbiamo esaminato come le proteine dell'isolato proteico di lenticchie rosse (RLPI) possano essere idrolizzate durante la fermentazione condotta con diversi ceppi di batteri lattici e lieviti. *Hanseniaspora uvarum* SY1 e *Fructilactobacillus sanfranciscensis* E10 sono risultati i microrganismi più efficaci nell'idrolisi delle proteine. *H.uvarum* SY1 ha prodotto i livelli più elevati di attività anti-radicalica, ACE-inibitoria e antifungina negli estratti solubili in acqua a basso peso molecolare (LMW-WSE). Abbiamo analizzato le 2039 sequenze peptidiche identificate nei LMW-WSE utilizzando il database BIOPEP UWM, e 36 sequenze corrispondevano a BPs noti. La fermentazione ha generato 12 peptidi non presenti nella RLPI non fermentata. Inoltre, *H.uvarum* SY1 ha prodotto le quantità più elevate di BPs, in particolare quelli con proprietà antiossidanti e ACE-inibitorie. I peptidi KVI, LVR e LVL sono stati identificati per la prima volta nei campioni fermentati. Inoltre, abbiamo trovato 44 nuovi potenziali BPs che mostravano attività antifungina e meritano ulteriori indagini e caratterizzazioni.

Key words: Lactic acid bacteria; yeasts; antiradical; ACE-inhibitory; antifungal, bioactive peptides, high resolution tandem mass spectrometry.

Introduction

Bioactive peptides production through microbial fermentation is considered by several authors (Gobbetti et al.;2007 De Pasquale et al., 2020) to be one of the most effective non-thermal, green biotechnology to exploit the full biological potential of different protein sources. It is a process that meets the requirements of sustainability, innovation and functionality, but in order to be effective it must necessarily be monitored and designed to obtain the metabolic pathways of interest (Tlais et al., 2021). This report highlights the main results of the three years of research activities of my PhD in Food Engineering and Biotechnology.
In particular, we investigated the release of bioactive peptides from proteins during fermentation of red lentils protein isolate (RLPI) with different microbial strains: *Lactiplantibacillus plantarum* LM1.3 (RLPI-LM1.3), *Lacticaseibacillus rhamnosus* ATCC53103 (RLPI-ATCC), *Fructilactibacillus sanfranciscensis* E10 (RLPI-E10), *Kazachstania unispora* KFBY1 (RLPI- KFBY1), and *Hanseniaspora uvarum* SY1 (RLPI- SY1). The fermented LMW peptides extracts were tested for different bioactivities including antiradical, ACE-inhibitory, and antifungal activities and further identified via high resolution tandem mass spectrometry (UHPLC-HRMS2).

Materials and methods

Red lentils protein isolate (50 g) and water (100 g) were mixed to form a fermentable dough with a dough yield (DY) of 300. Glucose (1%, w/w) was added to the dough, and it was then singly fermented using pure cultures of

LAB and yeast strains. After 24 hours, the cells were harvested by centrifugation at 10,000 rpm for 10 minutes at 4 °C, washed twice in 50 mM sterile potassium phosphate buffer (pH 7.0), and then inoculated into the RLPI dough. The final cell densities were approximately 7.0 Log CFU mL^{-1} for LAB and 5.0 Log CFU mL^{-1} for yeasts. The RLPI dough was fermented at 30 °C for 8 days. Two control samples were used: RLPI dough without bacterial inoculum and incubation (RLPI-Raw), and RLPI dough without inoculum but incubated at 30 °C (RLPI-Unstarted). To isolate the active peptide fraction, the water-soluble extract (WSE) was subjected to ultrafiltration with a molecular weight cut-off of less than 3 kDa, following the method described by Tagliazucchi et al. (2017) with some modifications. In this process, 15 mL of the sample was loaded into a Vivaspin®20 column with a 3000 MWCO-PES membrane (Sartorius, Italy) and centrifuged at 6000 rpm for 40 minutes. The resulting low molecular weight water-soluble extract (LMW-WSE) was used for further analysis. The identification of low molecular weight peptides in the LMW-WSE was performed using UHPLC/HR-MS2 (UHPLC Ultimate 3000, Thermo Scientific, San Jose, CA, USA; Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer, Thermo Scientific, San Jose, CA, USA) equipped with a C18 column (Zorbax SB-C18 Reversed-phase, 2.1 × 50 mm, 1.8 µm particle size, Agilent Technologies, Santa Clara, CA, USA), following the method described by Martini et al. (2020). The MS data was first converted into a .mgf file and then processed using the MASCOT software (Matrix Science, Boston, MA, USA) for peptide sequencing and identification. The identification process employed the following parameters: no enzyme specified, peptide mass tolerance of ±5 ppm, fragment mass tolerance of ±0.1 Da, and variable modifications including Deamidation (NQ), oxidation (M), and phosphorylation (ST). A maximum of one post-translational modification was permitted in a single peptide. Only peptides identified with a significance threshold of $P<0.05$ were considered for further analysis.

Results & Discussion

The inhibitory effects of raw and fermented Red Lentils Protein Isolate (RLPI) on the mold *Penicillium roqueforti* P1, a common bread spoilage agent, were evaluated using peptide extracts (LMW-WSPE). All LMW-WSE derived from fermented samples significantly enhanced the inhibition of radial hyphal growth rate in *P. roqueforti* P1 compared to RLPI-Raw (12.5 ± 1.52%). Notably, RLPI-SY1 exhibited the highest inhibitory activity (approximately 60.4 ± 0.7%), followed by RLPI-Unstarted (60.1 ± 1.06%), RLPI-LM1.3 (56 ± 1.06%), and RLPI-KFBY1 (54.2 ± 0.78%). The lowest inhibition of radial growth, relative to the control, was observed in RLPI-ATCC (50 ± 0.85%) and RLPI-E10 (35.4 ± 1.13%) (Figure 1A). Previous studies have confirmed the involvement of lactic acid bacteria (LAB), particularly *Lp. plantarum* and *Lc. rhamnosus*, with proteolytic activities in generating antifungal bioactive peptides against various *Penicillium* spp. However, there is currently no available literature data on the potential of *H. uvarum*, which exhibited the strongest inhibitory effect on *P. roqueforti* P1, in releasing antifungal peptides.

The renin-angiotensin system (RAS) plays a crucial role in regulating fluid balance and blood pressure, making it a significant metabolic process in cardiovascular homeostasis. Angiotensinogen, a protein primarily produced in the liver, is converted to angiotensin I (Ang I) by renin, an enzyme mainly secreted by the kidney. Angiotensin-converting enzyme (ACE), predominantly found in the lungs, converts Ang I to angiotensin II (Ang II), which has implications for human health (Marques et al., 2012). Evaluating the inhibitory effect on ACE activity is important for its potential impact on hypertension. In this study, an in vitro ACE-inhibitory activity assay was conducted to assess the antihypertensive potential of raw and fermented RLPI, and the results were reported as IC50, representing the amount of LMW-WSPE extract required to inhibit 50% of ACE activity. Among the samples, only RLPI fermented with *H. uvarum* SY1 (RLPI-SY1) exhibited significant ($P < 0.05$) ACE inhibition, with the lowest IC50 value (24.75 ± 0.77 µL) compared to RLPI-Raw (30.79 ± 0.87 µL) (Figure 1B). RLPI-Unstarted, RLPI-E10, and RLPI-KFBY1 showed similar IC50 values without statistical significance ($P > 0.05$) compared to RLPI-Raw. Notably, started fermentation had a significant negative effect ($P < 0.05$) on ACE inhibition in RLPI-LM1.3 (47.13 ± 0.97 µL) and RLPI-ATCC (33.76 ± 0.92 µL) (Figure 1B). Previous research has explored the potential inhibitory effect of red lentil protein hydrolysates, revealing the generation of bioactive peptides in RLPI hydrolyzed with commercial trypsin (Boye et al., 2010). Although the proteolytic systems of various lactic acid bacteria have been utilized to produce bioactive peptides with ACE-inhibitory activity through fermentation, none of the LAB starters used in our study were able to release anti-ACE peptides. In contrast, our findings demonstrated the effectiveness of *H. uvarum* SY1 in suppressing ACE activity. While yeast cells have been investigated as a source of bioactive peptides with ACE-inhibitory properties (Mirzaei et al., 2021), their use as starters to induce protein hydrolysis and release anti-ACE peptides has not been previously reported.

Antioxidant peptides have gained attention as a promising strategy for preventing oxidative stress and preserving food quality, thereby reducing economic losses in the food industry and improving public health (Chakrabarti et

al., 2014). In this study, the ABTS radical scavenging capacity of LMW-WSE from raw and fermented RLPI was evaluated. Compared to RLPI-Raw (2.12 ± 0.06 mM Trolox eq. g^{-1} DW), a significant increase ($P < 0.05$) in the ABTS radical scavenging capacity was observed in LMW-WSE of RLPI-SY1 (23.92 ± 0.01 mM Trolox eq. g^{-1} DW) and RLPI-Unstarted (6.36 ± 0.08 mM Trolox eq. g^{-1} DW). The thermal stability of the ABTS radical scavenging capacity of LMW-WSE from RLPI-SY1 was confirmed even after heat treatment at 100 °C for 5 minutes. Interestingly, the lactic acid fermentation had an unexpected negative effect on the antioxidant activity of RLPI (Figure 1C). This finding contradicted previous studies that highlighted the potential role of *Lp. plantarum* strains in cow's milk and *Lc. rhamnosus* in releasing antioxidant-rich hydrolysates and peptides (Aguilar-Toalá et al., 2017; Solieri et al., 2015).

In order to assess the impact of fermentations on peptide release, the peptide profile was examined using HPLC-HRMS analysis. Over the past few years, HRMS-based peptidomics analysis has emerged as a reliable and sensitive method for comprehensive mapping of the peptidome present in various samples. MS-based peptidomics analysis is particularly suitable for monitoring the abundance of known peptides in samples; however, its application in discovering novel bioactive peptides presents challenges due to the presence of numerous degradation products and inactive precursors (Aydoğan, 2020). The analysis revealed a total of 2039 distinct peptides across all samples, with only 391 peptides detected in RLPI-Raw. The variation in proteolytic activity associated with different starters had a significant impact on the quantity and diversity of peptides identified (Figure 2A). The use of lactic acid bacteria (LAB) led to the highest increase in the number of peptides, with RLPI-ATCC (1520 peptides) and RLPI-E10 (1506 peptides) exhibiting the greatest peptide diversity among all samples. Among yeast fermentations, RLPI-SY1 showed the highest diversity of peptide substrates, following RLPI-ATCC and RLPI-E10, while RLPI-KFBY1 displayed the lowest diversity. The influence of spontaneous fermentation on proteolytic activity was evident in RLPI-Unstarted, which showed a high number of distinct peptides (1421 peptides). The identified peptides in the analyzed samples were compared to known bioactive peptides (BPs) sequences using the BIOPEP UWM database. This database, which contains over 4600 BP sequences, has become popular in the field of food and nutrition science as a valuable source of data on these molecules, which are of great interest for potential use as functional food ingredients and nutraceutical applications (Minkiewicz et al., 2019). Most of the peptides found in both raw and fermented RLPI were related to parental proteins from chickpeas and a few other legumes. Out of the 2039 peptide sequences identified, a total of 36 peptides showed 100% identity with previously identified and validated BP sequences (Figure 2). The majority of these discovered BPs were associated with plant proteins. It is worth noting that most research on BPs has primarily focused on precursor proteins from dairy, meat, and fish, with a smaller portion dedicated to plant-based proteins (Bhat et al., 2015). Among these 36 BPs, 73% exhibited ACE-inhibitory activity, 22% had antioxidant activity, and 5% were associated with other bioactivities such as DPPIV-inhibition and antidiabetic properties. Thirteen of these BPs (ALEPDHR, FAP, FFI, KLP, LLP, LLPH, LNF, LVR, PLLR, PPP, TETWNPNHPEL, VVR, YLR), which were mainly ACE-inhibitory and antioxidant, were exclusively found in the fermented samples in varying amounts. Although the intensity of peaks varied, RLPI-SY1 had the highest number of BPs (36), followed by RLPI-Unstarted (35), while the remaining samples exhibited lower numbers ranging from 27 to 32. Furthermore, only 12 bioactive peptides were common among all the samples, with RLPI-Raw having the lowest diversity (Figure 2B). The impact of fermentation on the abundance of BPs was evident from the relative quantification analysis (Figure 2B and C). With few exceptions, each BP showed a considerable increase in peptide abundance compared to RLPI-Raw. RLPI-SY1 resulted in the highest cumulative intensity of BPs, followed by RLPI-Unstarted. Although LAB-fermented samples and RLPI-KFBY1 contained a higher number of BPs, their average abundance was relatively lower. RLPI-SY1 and RLPI-Unstarted were the richest sources of BPs compared to other treatments. The main bioactive peptides (BPs) that highly characterized RLPI-SY1 were ALEPDHR (antioxidant), AVV (ACE-inhibitory), FFI (ACE-inhibitory), FGG (ACE-inhibitory), KVI (antioxidant), LVL (ACE-inhibitory), LVR (ACE-inhibitory), and VVR (ACE-inhibitory). It is noteworthy that KVI, LVR, and LVL, which were the three most abundant peptides, were not previously obtained through fermentation. The presence of these BPs, along with other minor BPs, may contribute to the excellent functional properties observed in RLPI-SY1 during our screening. The main BPs highly present in RLPI-Unstarted were ALEPDHR, KAL (antioxidant), LAE (ACE-inhibitory), and PLLR (antioxidant). The other fermented samples exhibited different values of BPs, consistently higher than the control and lower than the two previously mentioned samples (Figure 2B and C). A correlation matrix based on the Spearman correlation coefficient was established to confirm the relationship between BPs and the screened bioactivities. The first correlation was made with the 36 BPs previously identified for their ACE-inhibitory and antioxidant activities. As expected, the majority of BPs showed a strong negative correlation with the IC50 values (indicative of ACE inhibition), with a few exceptions such as IAQ, KAL, LAA, LGF, and PLLR. Despite being classified for ACE inhibition in the literature, only PPP showed a substantially positive correlation with IC50 values. On the other hand, except for IAQ, KAL, LAA, PLLP, and PPP, all BPs were positively correlated

with antioxidant activity. As previously reported, the LMW-WSE of *H. uvarum* SY1 exhibited potent in vitro antioxidant and antihypertensive activity. The peptides KVI, LVL, and LVR, which were the most abundant peptides produced by RLPI-SY1, were strongly associated with anti-ACE and antioxidant activity. Additionally, several peptides (e.g., ADELPDHR, FAP, FFI, KLP, LLP, LLPH, LNF, and LVR) were exclusively found in the fermented sample and not in the raw substrate. These peptides also showed a strong correlation ($P < 0.05$) with anti-ACE and antioxidant activity. To gain more insight, we further investigated the relationships between the antifungal assay results and the 50 most prevalent peptides. Forty-four peptides showed a strong correlation with the inhibition of radial growth rate against P. roqueforti P1. Among the fermented and unfermented samples, it was found that RLPI-SY1 contained all these peptides in varying quantities. Notably, PSSSE, EPSSQS, HGPAP, HGAPP, INAENNQRNF, PSLSSM, PSISMS, TPSSEN, and VLVK were the most abundant peptides in RLPI-SY1 and were solely identified in the fermented RLPI. This discovery opens the door to further investigation, such as the ex-novo synthesis of these peptides, followed by validation of their bioactivity.

Conclusions

Under the experimental conditions of our study, we found that RLPI (rice protein isolate) was a suitable substrate for the release of bioactive peptides (BPs) through assisted-starter fermentation. The effectiveness of the proteolytic system of the microorganisms we investigated varied depending on the species. In particular, the yeast *H. uvarum* SY1, which has been underutilized in the release of BPs in food systems, showed promising results. Fermentation with H. uvarum SY1 led to an increase in ABTS-radical removal, ACE inhibition, and antifungal activities compared to raw RLPI. These activities were detected in the low molecular weight water-soluble extracts. The increase in activities was attributed to the release of specific BPs, some of which were previously unidentified in raw RLPI or fermented matrices. Additionally, we discovered 44 peptides that were correlated with antifungal activity for the first time, highlighting the need for further characterization of these peptides.

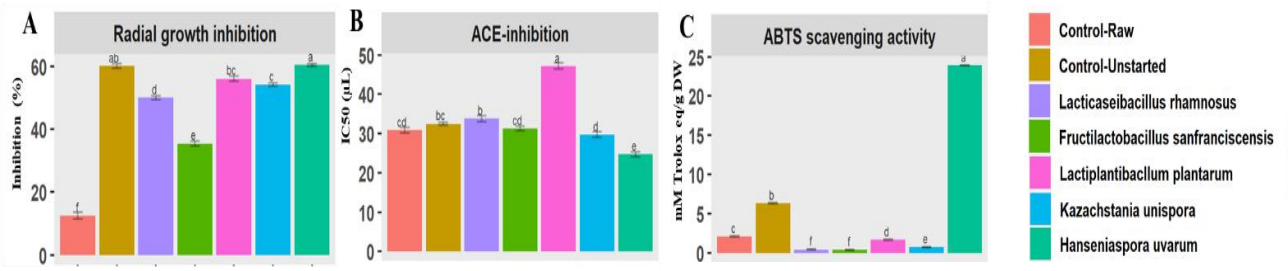

Figure 1. In vitro determination of radial growth inhibition against *Penicillium roqueforti* P1 (panel A), ACE-inhibitory (B), and ABTS scavenging activity of low molecular weight water soluble extracts (LMW-WSE) obtained from fermented and unfermented red lentils protein isolate.

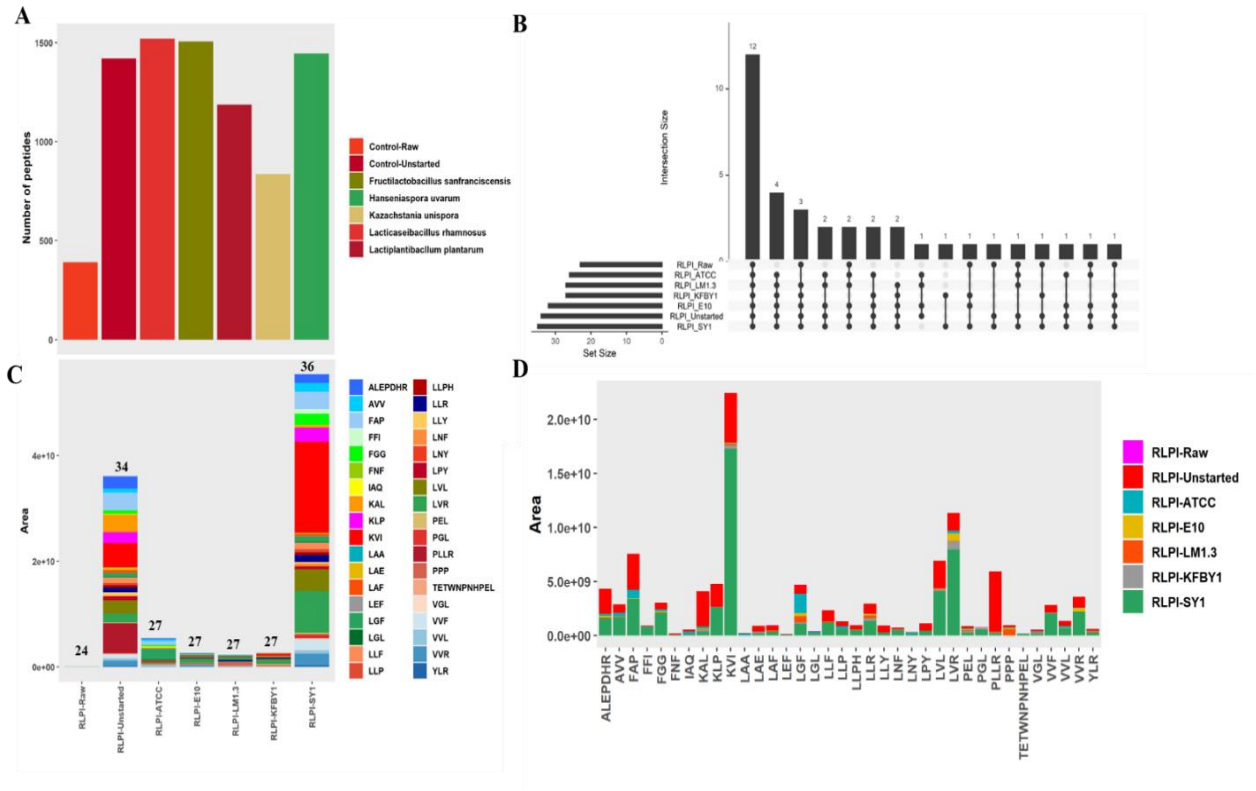

Figure 2. Peptidomic analyses of low molecular weight water soluble extracts (LMW-WSE) obtained from raw and fermented red lentils protein isolate. Total number of different peptides found in each sample (A); upset plot of the intersection of samples, sorted by identified BPs sharing 100% sequence homology with known bioactive peptides using BIOPEP UWM database, (dark circles in the matrix indicate sets that are part of the intersection) (B); relative quantification of BPs and distribution in the samples (C and D).

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Role of closure and oxygen dissolved at bottling on white wine evolution: a multiparametric approach

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The aim of this PhD project is to study the evolution of a bottled white wine sealed with different closures, characterised by different oxygen transfer rates (OTR). In addition, a recent innovative technology for winemaking, processing with contactor membrane, was used to manage dissolved gases at bottling, in particular oxygen.

Ruolo della chiusura e dell'ossigeno disciolto all'imbottigliamento nell'evoluzione di vini bianchi: un approccio multiparametrico

Il presente progetto di dottorato si propone di studiare l'evoluzione di un vino bianco imbottigliato mediante l'utilizzo di differenti chiusure, caratterizzate principalmente da diversi *oxygen transfer rate* (OTR). Inoltre, è stata applicata una recente innovazione nel mondo enologico, la membrana *contactor*, per la gestione dei gas disciolti, in particolar modo dell'ossigeno disciolto.

Key words: Contactor membrane; closure; oxidation; aroma; carbonyl compounds; cyclic voltammetry.

1. Introduction

The evolution of a bottled white wine is influenced by various parameters mainly related to the characteristics of the wine (Comuzzo *et al.* 2015; 2017; Kallithraka *et al.* 2009; Fracassetti *et al.* 2021), but especially by the packaging (bottle and closure) (Cantu *et al.* 2022; Lagorce-Tachon *et al.* 2016; Crouvisier-Urion *et al.* 2018) and storage conditions (Ferreira-Lima *et al.* 2013; Mas *et al.* 2002; Mafata *et al.* 2019; Wirth *et al.* 2012).
The International Organisation of vine (OIV) has authorised the use of contactor membranes (OIV-OENO 499-2013), which allow the management of dissolved gases (Schonenberger *et al.* 2019). This technique allows to reduce oxygen levels to concentrations below 0.2 mg/L (Schmidt *et al.* 2010). Contactors, potentially have a strong impact on evolution kinetics, while preserving the sensory and chemical characteristics of the wine.
In particular, the use of contactor membranes during the bottling phase would reduce the amount of dissolved oxygen, compensating the intense oxygenation related, for example, to filtration, which may contribute significantly to the amounts of oxygen dissolved at bottling (Day *et al.* 2015).
The management of oxygen in bottle is a critical factor, regulated by the performance of the closures (Silva *et al.* 2011; Skouroumounis *et al.* 2005; Godden *et al.* 2001). It is essential to carry out adequate maintenance of the bottling machine and, above all, to select a suitable closure with the appropriate oxygen transfer rate (OTR), for fulfilling a given winemaking project and producing wines with a good stability towards evolution.
Finally, in recent years, several research groups have studied new and faster analyses to determine the shelf-life of bottled white wine. In particular, cyclic voltammetry is a new technic in enology that can be useful in combination with different statistical approaches, to achieve this goal.

2. Materials and Methods

2.1 Sample Preparation

A Pinot Gris, vintage 2021, DOC delle Venezie, was selected for this research project. The wine was bottled at winery scale, using a GAI 3005 TOP integrated bottling machine (for technical cork closures), and a GAI 4292 corker (for screw caps) (GAI Macchine Imbottigliatrici Spa, Ceresole Alba, Italy). The wine was bottled in standard Bordeaux bottles, clear white, with a nominal volume of 750 mL at 20°C, with a BVS standard neck for the screwcaps.
Concerning oxygen management, two different treatments were applied to the wine: a control (no treatment) and a contactor membrane processing by using a Mastermind Remove equipment (Ju.Cla.S. S.r.l., Pescantina, Italy). Membrane had a nominal porosity of 0.05 µm and a MWCO < 50. The CO_2 concentration after the treatments was 1.5 g/L and 0.9 g/L respectively and the O_2 concentration was 1.2 mg/L in Control and 0.3 mg/L in membrane processed wine.
Four different closures were used for sealing the bottles: an agglomerated cork and three different screwcaps, one with a Saranex® liner, and two alternative liners named "M" and "Z", characterized by a lower OTR. Table 1 summarizes all the trials. Samples were stored at 20°C ±5°C and 70% ±10% relative humidity until analysis, carried out after three and eleven months.

Table 1 *Codes used for the different closures and estimated oxygen transfer rate.*

Sample code	Closure	Theoretical OTR
SUG	Cork	*n.k.*[1]
SAR	Saranex®	+++
LIN M	Liner "M"	++
LIN Z	Liner "Z"	+

[1] *n.k.: not known*

2.2 Oxygen dissolved

The oxygen dissolved in bottles was measured as described by Comuzzo et al. (2017).

2.3 Determination of Volatile Compounds

Volatile compounds were analysed by HS-SPME-GC-MS, using GC2030 Nexis gas chromatograph (Shimadzu, Kyoto, Japan) coupled with a QP2020NX mass spectrometer and an HT2800T autosampler (HTA S.r.l., Brescia, Italy). A divinylbenzene/carboxen/polydimethylsiloxane (DVB-CAR-PDMS, 50/30 µm x 2 cm) fiber (Supelco, Bellefonte, PA, USA) was used for SPME. Column was a DB-Wax 30 m x 0.25 mm i.d. x 0.25 µm (Agilent Technologies, Santa Clara, CA, USA) and the carrier gas was helium at a flow rate of 35 cm/s.

Ten millilitres of samples were added to 3 g of NaCl in 20 mL glass vials and immediately sealed. Ethyl heptanoate (Sigma-Aldrich, St. Louis, MO, USA) was used as internal standard at 2.17 mg/L. The analysis was carried out setting the following conditions: incubation for 15 minutes at 40 °C, under stirring (500 rpm 5 seconds on and 2 seconds off), microextraction for 15 minutes, desorption for 5 minutes of the fiber into the injector, in splitless mode. The initial oven temperature was 40 °C held for 5 minutes; then it was ramped at 4 °C/min up to 240 °C, held for 15 minutes. For the qualitative analysis, mass spectra were acquired at 70 eV and compared with those reported in the NIST 20 mass spectra library. Moreover, linear retention indices were calculated, using *n*-alkanes (C_7-C_{30}) (Sigma-Aldrich, St. Louis, MO, USA), and compared with those found in literature. Finally, for some of the detected compounds identification was confirmed by comparison with commercial standards. Semi-quantitative analysis was carried out by the internal standard method.

For carbonyl compounds, the method proposed by Moreira *et al.* (2019), based on *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) derivatisation, was used with some modifications. Internal standard was *p*-fluorobenzaldehyde at 0.64 mg/L in wine (Sigma-Aldrich, St. Louis, MO, USA). Chromatographic conditions were as above, while microextraction was held for 45 min.

Table 2 *Composition of the chemical classes in which volatile compounds were grouped.*

Ethyl esters	Acetic esters	Other esters	Ageing markers	Alcohols	Acids
Ethyl butanoate	3-methyl-1-butanol acetate	Isopentyl hexanoate	Diethyl succinate	2-methyl-1-Propanol	2-methyl-propanoic acid
Ethyl hexanoate	Hexyl acetate	Propyl octanoate	Ethyl 9-decenoate	3-methyl-1-butanol	Butanoic acid
Ethyl octanoate	2-phenylethyl acetate	Caprylic acid, isobutyl ester		1-Hexanol	Hexanoic acid
Ethyl decanoate		Octanoic acid, 3-methylbutyl ester		1-Octanol	Octanoic acid
Ethyl dodecanoate				1-Decanol	Decanoic acid
Ethyl tetradecanoate				2-phenylethanol	
Ethyl hexadecanoate					

2.4 Cyclic voltammetry

Cyclic voltammetry was performed by the setup proposed by Comuzzo et al. (2017), with a modification. For the electrochemical analysis, screen-printed Metrohm 110 was used (Metrohm Italiana Srl, Origgio, Italy).

2.5 Statistical Analysis

Statistical analysis was performed with software Statistica 8 (Statsoft Inc., Tulsa, OK, USA) to obtain statistical significance using a two-way ANOVA followed by Tukey's post-hoc test; R 4.2.1 (R Development Core Team) to perform the Principal Component Analysis (PCA).

3. Results and Discussion

3.1 Dissolved oxygen

Dissolved oxygen was measured three days after bottling in order to allow the equilibration of the wines and repeated every two months.

The first measurement showed a significant difference between membrane processed and control samples, except for the wines sealed with technical cork (Figure 1). In the following 95 days, the samples closed with screwcaps showed a slower oxygen consumption with respect to those closed with cork. From day 144 to 424, oxygen concentration reached a stability and screwcap had lower levels with respect to cork closures, with similar behavior, probably due to their lower OTR.

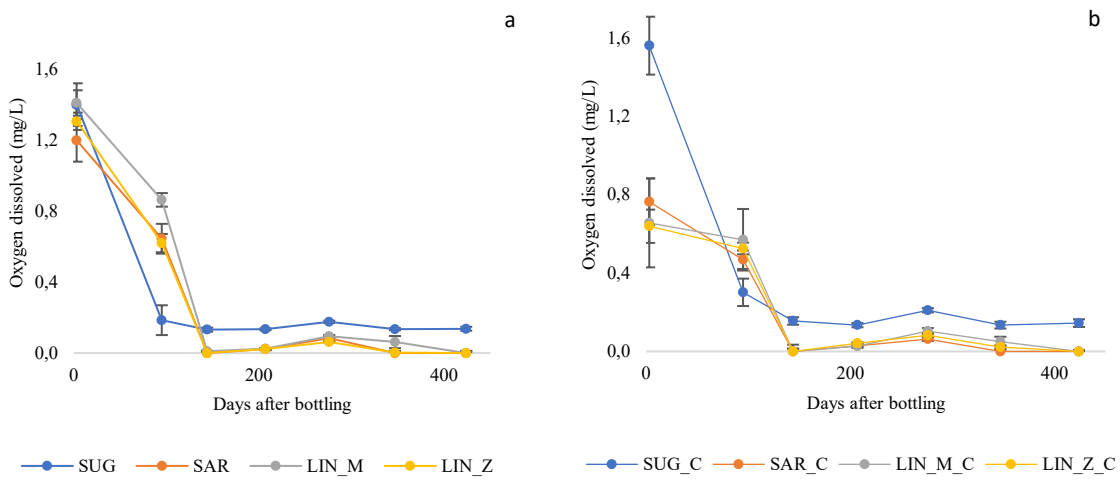

Figure 1 *Kinetic of oxygen consumption after bottling, in (a) control samples and (b) samples treated by contactor membrane.*

3.2 Aroma Compounds (ACs)

The characterisation of the volatile composition (Table 3) registered significant differences only for ethyl esters at the first sampling point, after three months of storage. This difference is mainly related to ethyl octanoate (Table 4); this ester was which is the most abundant among ethyl esters and its concentration was higher in the samples treated with the contactor membrane. The same trend was observed for the other aroma groups (Table 3), even if without significant differences.

After eleven months of bottle storage, statistical differences among samples increased, particularly for ethyl esters, ageing compounds and organic acids, with a generalized lower amount in the membrane-treated samples. Between the two different sampling points, there was a strong decrease in acetic esters, organic acids and other esters. Instead, there was a decrease of ageing compounds after membrane processing, except for Saranex. Table 4 summarizes the molecules that changed the most over time, between the two sampling points.

Table 3 *Volatile composition of the samples after 3 and 11 months (ACs divided in groups as in Table 2; values in µg/L). Different letters indicate significant differences between samples of the same sampling point to ANOVA e Tukey HSD test (p <0,05).*

Compounds	Time (months)	SUG		SUG_C		SAR		SAR_C		LIN_M		LIN_M_C		LIN_Z		LIN_Z_C	
Ethyl esters	3	2552,4 ± 87,83	ab	2776,35 ± 223,12	b	2437,57 ± 322,86	ab	2852,53 ± 265,66	b	2595,18 ± 227,37	ab	2531,39 ± 62,91	ab	2142,97 ± 225,58	a	2292,12 ± 73,89	
	11	2380,49 ± 253,09	ab	2311,68 ± 109,61	a	2686,79 ± 62,56	ab	2772,57 ± 39,84	ab	2469,18 ± 111,33	ab	2453,07 ± 70,53	b	2492,01 ± 106,64	ab	2585,31 ± 220,85	
Acetic esters	3	682,74 ± 52,62	a	787,16 ± 73,84	a	738,71 ± 72,59	a	800,37 ± 24,09	a	736,25 ± 51,87	a	726,27 ± 29,62	a	693,84 ± 15,01	a	716,06 ± 26,59	
	11	390,48 ± 18,34	a	389,56 ± 19,45	a	384,77 ± 8,73	a	397,46 ± 12,09	a	386,86 ± 19,04	a	400,32 ± 32,83	a	383,91 ± 9,21	a	397,52 ± 30,47	
Other esters	3	22,25 ± 0,78	a	23,3 ± 2,79	a	18,95 ± 5,24	a	23,7 ± 3,78	a	21,29 ± 1,39	a	20,95 ± 1,43	a	16,63 ± 2,71	a	17,86 ± 2,23	
	11	15,18 ± 3,06	a	13,78 ± 0,66	a	15,65 ± 1,11	a	17,08 ± 2	a	14,67 ± 1,27	a	15,33 ± 0,31	a	14,83 ± 0,32	a	15,28 ± 1,12	
Ageing compounds	3	6,16 ± 0,97	a	8,07 ± 1,42	a	5,31 ± 0,25	a	7,54 ± 1,68	a	6,95 ± 0,9	a	9,02 ± 3,39	a	5,27 ± 0,7	a	5,58 ± 0,53	
	11	15,45 ± 2,05	ab	12,19 ± 0,51	a	16,85 ± 0,12	b	16,82 ± 0,34	b	16,53 ± 1,43	b	15,56 ± 0,4	ab	16,52 ± 1,87	b	14,9 ± 2,73	
Alcohols	3	371,07 ± 16,89	a	407,44 ± 48,97	a	364,73 ± 15,83	a	412,56 ± 17,5	a	399,2 ± 31,9	a	385,43 ± 24,41	a	370,78 ± 7,04	a	375,33 ± 19,45	
	11	356,03 ± 15,19	a	331,53 ± 20,84	a	377,41 ± 28,66	a	368,02 ± 28,77	a	379,24 ± 28,79	a	365,72 ± 36,9	a	361,64 ± 31,46	a	349,52 ± 58,82	
Organic acids	3	124,87 ± 7,85	a	168,86 ± 20,5	a	140,32 ± 5,5	a	171,64 ± 29,16	a	162,41 ± 22,44	a	148,9 ± 20,93	a	128,15 ± 8,42	a	134,93 ± 19,42	
	11	99,84 ± 12,54	ab	80,3 ± 3,53	a	121,44 ± 1,95	b	124,23 ± 6,65	b	111,88 ± 8,38	b	105,99 ± 5,44	ab	116,61 ± 14,85	b	104,06 ± 19,22	

*concentration in µg/L

Table 4 *ACs with the greatest variation between 3 and 11 months (ACs divided in groups as in Table 2; values in µg/L). Different letters indicate significant differences between samples of the same sampling point to ANOVA e Tukey HSD test (p <0,05).*

Compounds	Time (months)	SUG		SUG_C		SAR		SAR_C		LIN_M		LIN_M_C		LIN_Z		LIN_Z_C	
Ethyl octanoate	3	1658,44 ± 57,63	ab	1784,22 ± 152,91	b	1569,53 ± 201,02	ab	1791,57 ± 147,29	b	1673,48 ± 153,06	ab	1615,05 ± 44,78	ab	1395,17 ± 113,05	a	1444,49 ± 58,6	ab
	11	1573,24 ± 163,37	a	1536,51 ± 80,1	a	1758,9 ± 30,21	a	1789,84 ± 14,29	a	1582,13 ± 77,89	a	1566,1 ± 42,55	a	1611,97 ± 73,68	a	1665,42 ± 142,79	a
3-methyl-1-butanol acetate	3	497,14 ± 47,24	ab	581,47 ± 50,66	b	485,61 ± 31,07	a	577,49 ± 19,42	ab	540,34 ± 31,51	ab	531,55 ± 24,71	ab	512,52 ± 13,21	ab	523,37 ± 24,42	ab
	11	311 ± 15,08	a	310,74 ± 17,14	a	297,73 ± 6,11	a	303,28 ± 8,58	a	302,64 ± 13,41	a	312,77 ± 28,55	a	298,84 ± 6,03	a	307,01 ± 23,63	a
Hexyl acetate	3	170,85 ± 6,48	a	185,53 ± 20,7	a	236,76 ± 103,5	a	202,79 ± 2,99	a	176,96 ± 19,23	a	178,5 ± 4,05	a	166,5 ± 2,85	a	176,66 ± 1,43	a
	11	73,55 ± 2,67	a	74,57 ± 2,59	a	80,45 ± 2,96	ab	87,32 ± 3,65	b	78,07 ± 5,53	ab	81,71 ± 4,2	ab	78,48 ± 2,51	ab	84,66 ± 6,07	ab
2-phenylethyl acetate	3	14,75 ± 0,19	a	20,17 ± 2,95	a	16,34 ± 0,24	a	20,09 ± 2,8	a	18,94 ± 2,32	a	16,21 ± 2,51	a	14,81 ± 0,79	a	16,04 ± 2,77	a
	11	5,92 ± 0,92	ab	4,25 ± 0,29	a	6,59 ± 0,08	b	6,86 ± 0,49	b	6,14 ± 0,42	ab	5,84 ± 0,12	ab	6,6 ± 0,87	b	5,85 ± 1,2	ab
Diethyl succinate	3	2,17 ± 0,48	a	2,84 ± 0,6	a	2,19 ± 0,2	a	2,44 ± 0,41	a	2,44 ± 0,35	a	2,31 ± 0,37	a	2,11 ± 0,07	a	2,26 ± 0,45	a
	11	13,01 ± 1,48	a	10,29 ± 0,39	a	13,7 ± 0,34	a	13,62 ± 0,44	a	13,74 ± 1,24	a	12,95 ± 0,38	a	13,67 ± 1,6	a	12,17 ± 2,29	a
2-phenylethanol	3	34,16 ± 1,71	a	42,55 ± 4,47	a	35,32 ± 1,73	a	41,28 ± 6,61	a	38,55 ± 3,45	a	36,17 ± 4,56	a	31,87 ± 1,79	a	34,21 ± 4,98	a
	11	25,99 ± 2,15	b	18,9 ± 0,45	a	27,26 ± 1,31	b	28,87 ± 1,8	b	27,67 ± 2	b	26,28 ± 0,48	b	27,9 ± 3,23	b	24,32 ± 4,53	ab
Octanoic acid	3	76,02 ± 5,26	a	103,39 ± 11,66	a	84,32 ± 3,22	a	102,15 ± 17,52	a	98,3 ± 13,63	a	86,11 ± 11,64	a	77,23 ± 5,93	a	81,04 ± 13,47	a
	11	61,01 ± 8,07	ab	48,82 ± 2,01	a	73,75 ± 1,15	b	75,2 ± 4,58	b	67,13 ± 4,88	b	63,56 ± 3,28	ab	70,33 ± 8,97	b	62,62 ± 10,19	ab

*concentration in µg/L

3.3 Cyclic voltammetry

The values of current intensity (μA) of the anodic trace of the cyclic voltammograms were analysed by PCA. At the first sampling point, samples showed the tendency to have a slightly higher curve with the application of the contactor membrane (Figure 2). This could be related to the lower level of dissolved oxygen at bottling, and thus to the greater preservation of the phenolic fraction.

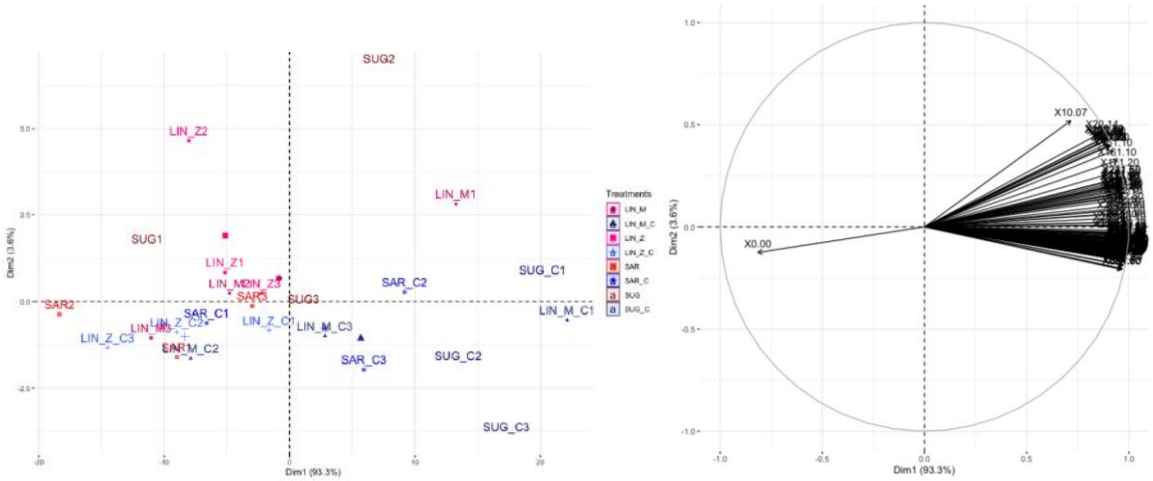

Figure 2 *Principal Component Analysis (PCA) on anodic trace data of cyclic voltammetry, after three months of bottle storage. For each sample, data from triplicate analysis were used.*

3.4 Carbonyl compounds

The method for the quantification of carbonyl compounds, developed by Moreira et al. (2019) for Porto wines, demonstrated to be a useful tool for the analysis of wine evolution.

The carbonyl compounds detected in this work are: hexanal (herbaceous), 2- and 3-methylbutanal (fruity odor), propanone (solvent) and phenylacetaldehyde (honey).

In Figure 3, it can be seen that liner "M" and Saranex, treated with a contactor membrane to manage gases, contain a lower amount of carbonyl compounds. This effect could be related to a lower concentration of oxygen during the storage period. This behaviour does not appear for the liner "Z", and this could be related to a greater rigidity of the liner; if this hypothesis will be confirmed in future experiments, the sealing properties of this liner could be improved by a higher pressure applied at corking.

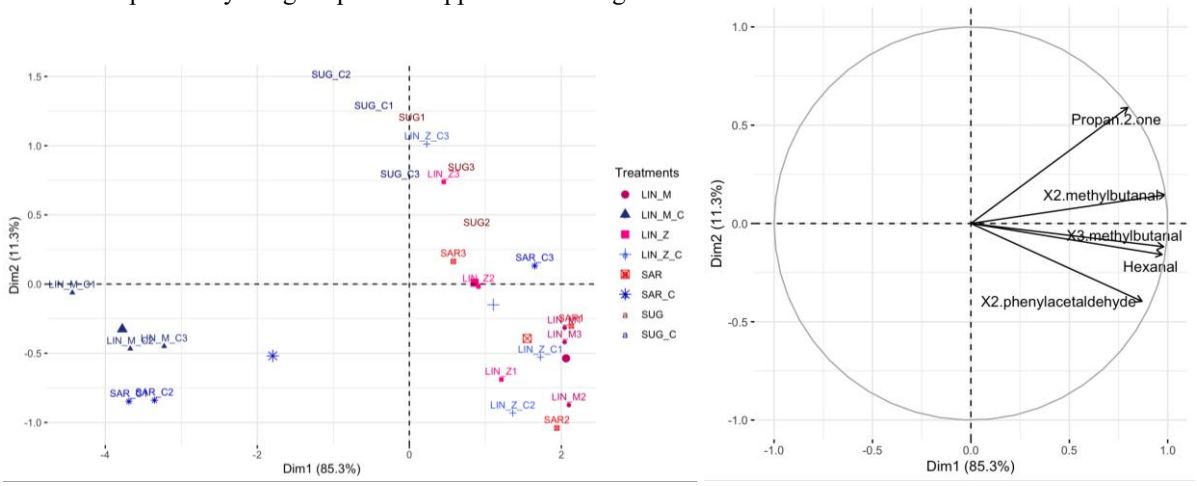

Figure 3 *Principal Component Analysis (PCA) based on absolute areas detected for carbonyl compounds, after 11 months of bottle storage. For each sample, data from triplicate analysis were used.*

4. Conclusions and Future Perspectives

Results demonstrated that the use of contactor membrane could help to reduce the initial concentration of dissolved gases, especially oxygen, allowing a better evolution of the wine over time. This technique, combined with an appropriate closure selection, can improve the evolution of white wines.

Further analysis will be carried out on the data collected in terms of chemical composition, electrochemical and sensory data; moreover, additional samplings will be performed to better understand the mechanisms involved in the evolution of the different samples.

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Technological approaches to improve the quality of meat products

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This research project dealt with the feasibility of using the Olive Leaf Extract (OLE) to limit the amount of nitrate and nitrite added in ripened sausages. The research activity has been developed, during the three years of the Ph. D course, in three steps: i) preliminary trial of ripened sausages production with different nitrate/nitrite and OLE ratios carried out at laboratory scale; ii) production of sausages with OLE added at industrial scale (*scale-up*); iii) evaluation of shelf-life of ripened sausages with OLE during MAP storage at 4°C.

Approcci tecnologici per migliorare la qualità dei prodotti carnei

Questo progetto di ricerca riguarda la possibilità di utilizzare l'estratto di foglie di olivo (OLE) per ridurre la presenza dei nitrati e nitriti nelle salsicce stagionate, responsabili della formazione dei composti N-nitroso. Il progetto è articolato in tre diverse fasi, tra cui: i) studio preliminare di produzione delle salsicce stagionate con diversi rapporti di nitrati/nitriti e OLE su scala di laboratorio; ii) produzione delle salsicce stagionate addizionate di OLE su scala industriale; iii) valutazione della shelf-life delle salsicce stagionate addizionate di OLE durante la conservazione in MAP a 4°C.

Key words: vegetable extract; nitrate and nitrite reduction; additives; ripened sausages; shelf-life evaluation.

1. Introduction

Meat and meat products have an important role in human nutrition since they are source of noble proteins, provide all essential amino acids and various micronutrients. They are an iron source in the bio-available form and, therefore, better absorbable by human body, good levels of vitamins B6 and B12, vitamin D (Ferguson, 2010). Furthermore, they are part of the eno-gastronomic culture of several countries. However, medical and scientific studies have correlated the consumption of these products, especially of red and processed meat, with the development of various diseases in the cardiovascular system and to an increase in the risk of cancer (Turner and Lloyd, 2017). In fact, the high-fat intake, and/or carcinogens compounds generated through processing methods and the additive transformation may be responsible of these risks. Among meat additives, nitrate and nitrite are used to allow stability of the red colour, inhibition of the growth of undesirable bacteria, improvement of the oxidative stability and contribution to flavour formation (Flores and Toldrá, 2021). Nevertheless, the addition of these synthetic additives to processed meat can induce the development of N-nitroso compounds linked with genotoxicity and metabolic disturbances in the intestine mucosa, with the subsequent risk to potentially develop colorectal cancer (Jian *et al.,* 2019). For these reasons, over the years, the replacement of nitrate and nitrite by natural extracts in processed meat have been proposed as valuable alternative. Natural ingredients, essential oils, extracts from fruits and vegetables spices have been used as nitrite scavenging and inhibitors of N-nitroso compounds formation, such as nitrosamines (NAs) (Tian *et al.,* 2020). Furthermore, extracts from wastes and by-products of the agricultural and food industry sectors contain highly valuable bioactive substances such as polyphenols with antioxidant and antimicrobial activity. Thus, olive leaves have been proposed for food preservation thanks to the abundant bioactive molecules with antioxidant and antimicrobial activities, such as oleuropein (Difonzo *et al.,* 2017). Several studies showed that the addition of OLE improved the oxidative stability, exerted an antimicrobial effect, and extended the shelf-life of several foods. However, its application on meat-based products has been less studied. In the present oral communication, the main results of the feasibility of using OLE for the reduction of nitrate and nitrite level in the ripened pork sausages are reported. This objective has been developed according to three different experimental steps: A1) preliminary trial of ripened sausages production with OLE addition at laboratory scale; A2) production of ripened sausages with OLE added at industrial scale (*scale-up*); A3) shelf-life prediction of ripened sausages with OLE during storage in MAP condition at 4°C.

2. Material and Methods

2.1 Preliminary trial of ripened sausages production with OLE addition in laboratory scale

For the preliminary trials in laboratory scale, an OLE extract, obtained with the procedure described in Difonzo *et al.* (2017) has been used. Pork meat was purchased from a local farm Salumi Martina Franca S.r.l. (Martina Franca, Italy). Sausages were manufactured using the lean meat and the adipose tissue (85/15, w/w) from extensively reared pigs. Potassium nitrate E252, sodium nitrite E250 (SolMar, Italy) and salt (40 g/kg of raw meat) were added. Six different formulations of sausages (F) with different nitrate, nitrite and OLE ratios were

produced and compared to a control sample containing the maximum levels of nitrate and nitrite admitted by Reg. (UE) No 1129/2011. Control (OLE: 0 mg/kg; NO_2-NO_3: 150-150 mg/kg); F1 (OLE: 200 mg/kg; NO_2-NO_3: 75-75 mg/kg); F2 (OLE: 400 mg/kg; NO_2-NO_3: 75-75 mg/kg); F3 (OLE: 800 mg/kg; NO_2-NO_3: 75-75 mg/kg); F4 (OLE: 200 mg/kg; NO_2-NO_3: 0 mg/kg); F5 (OLE: 400 mg/kg; NO_2-NO_3: 0 mg/kg), F6 (OLE: 800 mg/kg; NO_2-NO_3: 0 mg/kg). After ripening the nitrate and nitrite residual content, sulphite *Clostridia* and spores, *Coliforms, Escherichia coli* and *Staphylococcus* coagulase positive, were carried out following the methods described in Difonzo *et al.* (2022).

2.2 Production of ripened sausages with OLE added at industrial scale (*scale-up*)

Based on the results obtained from the first experimental trials, a *scale-up* approach was also performed. In this case, a commercial OLE (Olive Leaf Extract, Hepatica, Germany) commonly marketed as dietary supplement, at 40% of oleuropein content, was used for the production Ripened sausages were manufactured at the local farm Salumi Martina Franca S.r.l. sited in Martina Franca (Taranto, Italy) following common industrial processing applied by the company. The raw meats were minced by an industrial meat grinder equipped with a pre-mixer. In this case, five different formulations (F) with different nitrate, nitrite and OLE ratios were considered (Table 1).

Table 1 *Formulations of OLE – nitrate and nitrite used for samples preparation during the scale-up.*

Sample	F1	F2	F3	F4	F5
Olive Leaf Extract (mg/kg)	1000	1000	0	0	500
Nitrate and nitrite (mg/kg)	0	75 NO_2 75 NO_3	0	75 NO_2 75 NO_3	35 NO_2 35 NO_3

During mechanically kneading, salt (20 g/kg) and pepper (1 g/kg) were also added. The mixture was mechanically stuffed into natural pork casings and submitted to stewing (23°C, RH 95%, 24 h) to activate the fermentation process, drying (17-20 °C, RH 60-75%, 96 h) and ripening (15-18 °C, RH 80%). At the end of ripening period, physicochemical analysis, colour parameters, lipid oxidation and nitrosamines content were evaluated.

2.2.1 Weight loss, water activity (a_w), pH, moisture content

Weight loss (%) was calculated as percentage of differences in weight of the whole sausages between day 0 and the end of ripening time. The pH was measured by inserting the glass pH electrode in the meat portion (HANNA instruments, Woonsocket, USA) and the a_w was determined using a hygrometer (Aqua Lab 100–240 V AC, Pullman, USA). The determination of moisture content (%) was carried out according to AOAC International methods 950.46 (AOAC, 2006).

2.2.2 Colour measurement

The colour of ripened sausages was analysed according to the International Lighting Commission (CIE) system using the parameters L^* (lightness), a^* (redness), b^* (yellowness) with a colorimeter CM-600d (Konica Minolta, Tokyo, Japan) equipped with the software SpectraMagic NX (Konica Minolta, Tokyo, Japan). The measurements were taken on different points on both surfaces of three 2-cm-thick slices of each sausage.

2.2.3 Lipid oxidation

The lipid oxidation level of ripened sausages was determined by thiobarbituric acid reactive substances (TBARs) as reported in Rosmini *et al.* (1996). The results were expressed in mg of malondialdehyde (MDA) per kg of ripened sausages, using a calibration curve obtained using 1,1,3,3-Tetraethoxypropane (TEP) as standard (Sigma-Aldrich, Germany) (0.01 M) at different concentrations (0.22 mg/kg - 2.2 mg/kg).

2.2.4 Nitrosamines content

Nitrosamines were extracted according to the method described by Cintya *et al.* (2019). The NAs level was determined using Ultra High Performance Liquid Chromatography (UHPLC) (Thermo Fischer Scientific, Waltham, USA) as reported in Al-kaseem *et al.* (2014). Wavelengths of 231 nm was used for absorbance detection and the quantification of the nitrosamines was obtained using the EPA 521 standard (Nitrosamine Mix, Supelco, Bellefonte, USA). The results were expressed in µg/kg of ripened sausage.

2.2.5 Statistical analysis

Data were subjected to Two-Way ANOVA followed by the Tukey's HSD test considering the dose of OLE and the dose of nitrate and nitrite added as independent variables. Significant differences were determined at $p<0.05$ by Minitab Statistical Software (Minitab Inc., State College, USA).

2.3 Shelf-life prediction of ripened sausages with OLE during storage in MAP condition at 4°C

The extension of shelf-life of the meat-based products is another challenge of the meat industry and ripened sausages were increasingly marketed in vacuum or in Protected Atmospheres Packaging (MAP), as whole piece

or sliced. MAP is one of the preservation and packaging solutions being employed to meet customer demand for food and its use for processed meat has greatly grown in recent years (Ameer *et al.,* 2022). In the third experimental step of this research a shelf-life study of ripened sausages with OLE added was performed. To this aim, samples F1, F4 and F5 (Table 1) carried out during *scale-up* were considered. In this way, the effect of OLE when it is added alone and in combination with nitrates and nitrites in comparison to a sample with only synthetic additives added should be highlighted during the storage. At the end of ripening period, sausages were sliced, placed in sterile plastic trays (95 × 10 mm), and packed in MAP condition using a gas mixture 70:30 N_2/CO_2 and a plastic film composed of orientated polyamide/polypropylene (OPA/PP). All packs were stored at 4 °C for 80 days and examined at 0 days (T0), 10 days (T10), 20 days (T20), 40 days (T40), 60 days (T60) and 80 days (T80). The evolution of the oxidative degradation carried out with TBARs-test was considered as target phenomena to predict shelf-life of ripened sausages with OLE.

3. Results and Discussion

3.1 Preliminary trial of ripened sausages production with OLE addition in laboratory scale

Microbiological analysis, nitrate and nitrite residual content

The aims of the preliminary trials were to assess the hygiene and safety parameters and the nitrate residual content in the different formulation. At the end of ripening period in all sausages with OLE added, all the microbiological parameters evaluated were within the law limits Reg. (CE) No. 1441/2007 (data not shown). A possible role of OLE in exerting an antimicrobial effect can be assumed since also in the samples without synthetic additives no growth was found. This could be due to the direct inhibitory action of OLE and the presence of polyphenolic compounds with antimicrobial properties. Furthermore, an effectiveness reduction of residual nitrate and nitrite level was obtained. In fact, the highest residual values were found in the control sample with only nitrate and nitrite. On the contrary, in the samples with only OLE added residual nitrate and nitrite were lower than limit of detection or not detected (data not shown).

3.2 Ripened sausages with OLE added produced during *scale-up*

The main aims of this second study were the standardisation of the production process of ripened sausages and to highlight the evaluation of OLE addition on the lipid oxidation and the presence of NAs at the end of ripening period, considering the production with industrial equipment.

Weight loss, water activity (a_w), pH, moisture content

The results of weight loss (%), moisture (%), pH and a_w of the ripened sausages are shown in Table 2. As regards moisture and activity water (a_w), the presence of nitrate and nitrite in samples F2, F4 and F5 resulted in a significant reduction compared to samples F1 and F3 with only OLE and without any type of additives, respectively. This could be due to the osmotic dehydration induced by the presence of these synthetic additives (Deng *et al.,* 2021).

Table 2 *Mean value, standard deviation, and results of the statistical analysis (two-way ANOVA) of weight loss, moisture, pH and water activity (a_w) of the ripened sausages.*

	F1	F2	F3	F4	F5	*p-value*
Weight loss (%)	41.61±0.42^B	48.56±0.44^A	42.03±1.04^B	48.08±1.18^A	47.85±1.00^A	OLE =0.056 NO_2-NO_3=0.008
Moisture (%)	29.85±0.65^A	24.60±0.27^B	30.51±1.00^A	22.46±0.38^C	23.88±0.89^B	OLE=0.057 NO_2-NO_3<0.001
pH	6.04±0.02^B	6.04±0.03^B	6.13±0.02^A	6.18±0.03^A	6.02±0.02^B	OLE=0.003 NO_2-NO_3<0.001
a_w	0.85±0.01^A	0.83±0.01^B	0.86±0.00^A	0.83±0.00^B	0.82±0.01^B	OLE=0.081 NO_2-NO_3<0.001

Different letters in the same raw indicate significant differences at $p<0.05$.

This trend has been also confirmed by weight loss which is generally related to these two latter parameters. In fact, the presence of nitrate and nitrite, both alone (F4) and in combination with the OLE (F2, F5) resulted in significantly higher weight loss than the samples where they were not added. Considering pH, the presence of the OLE (F1, F2, F5) resulted in a significant decrease of this parameter. This could be due to the presence of fermentable carbohydrates of the OLE that could promote the metabolism of lactic acid bacteria which induce acidification (Flamminii *et al.,* 2019).

Colour analysis

The results of colour analysis are reported in Table 3. The absence of nitrate and nitrite in samples F1 and F3 induced a significant reduction of *a** index, probably due to the lack of synthesis of the nitroso-myoglobin which determine the stability of the typical red colour of these products. On the contrary, sample F4 with only with nitrate and nitrite showed significantly higher value than all other samples.

Table 3 *Mean value, standard deviation, and results of the statistical analysis (two-way ANOVA) of lightness (L*), redness (a*) and yellowness (b*) of the ripened sausages.*

	F1	F2	F3	F4	F5	*p-value*
Lightness (*L)**	36.61±0.24^{A}	34.18±0.08^{B}	36.37±0.07^{A}	33.59±0.29^{C}	34.68±0.42^{B}	OLE=0.104 NO_2-NO_3<0.001
Redness (*a)**	6.43±0.69^{D}	8.07±0.18^{B}	5.74±0.16^{D}	8.78±1.39^{A}	6.87±1.05^{C}	OLE=0.085 NO_2-NO_3<0.001
Yellowness (*b)**	6.85±0.42^{BC}	6.64±0.27^{C}	8.10±0.92^{A}	7.65±0.64^{B}	6.63±0.81^{C}	OLE=0.144 NO_2-NO_3=0.033

Different letters in the same raw indicate significant differences at $p<0.05$.

Considering yellowness (*b**), generally associated with lipid oxidation, sample F3 without any type of additive added showed significantly higher value than all other samples. On the contrary, sample F2 and F5 with both types of additives at different concentration showed significantly lower values. Furthermore, no significant differences emerged compared to F1 with only OLE added. Differences emerged among different formulations could be due to the different lipid oxidation level of ripened sausages which could be limited by the presence of OLE both alone (F1) and in combination with nitrate and nitrite (F2, F5).

TBARs-test

The antioxidant effect of the OLE was also highlighted by TBARs-test results reported in Figure 1. Sample F3 was the most oxidised with an MDA mean content of 0.66 mg/kg sample. In contrast, sample F2 with OLE and nitrate/nitrite at the highest doses showed the lowest value. In addition, sample F1 with only OLE showed significantly lower MDA content than sample F4 with only nitrate and nitrite. These results therefore could confirm the effectiveness of OLE both alone and in combination with nitrate and nitrite in retarding the oxidation process of the ripened sausages thanks to the antioxidant effect of OLE, particularly richness in oleuropein which can act as scavenger against free radicals (Hassen *et al.,* 2015).

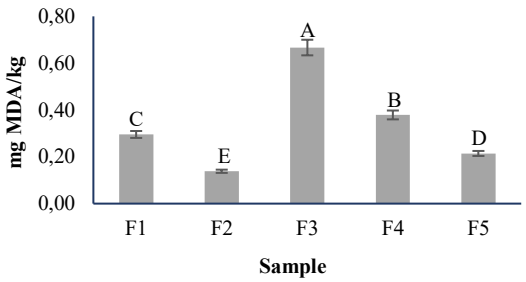

Figure 1 *Results of the TBARs-test (mg MDA/kg) of the ripened sausages. Different letters indicate significant differences at p<0.05.*

Nitrosamine content

In Figure 2 the total nitrosamines content is reported. As it was expected the highest significant value was found in sample F4 with only nitrate and nitrite. On the contrary the lower values were found in samples F1 and F3 without synthetic additives added. The reduction of the OLE and nitrate/nitrite doses in sample F5 induced a significant reduction compared to sample F2 at the highest doses. These results could highlight the effectiveness of the extract in the reduction of nitrosamines formation in sausages at the end of the ripening period. In fact, it was demonstrate that polyphenols could reduce nitrite to hydroxyl groups in their structure to release hydrogen to react with free radical, blocking the chain reaction of free radicals, thus reducing the formation of nitrosamines (Gao *et al.,* 2022). At the same time, although at significant lower amount, the presence of these compounds in samples F1 and F3, could be due to the presence of the OLE which naturally present little amount of nitrite and contamination events that occurred during processing, respectively.

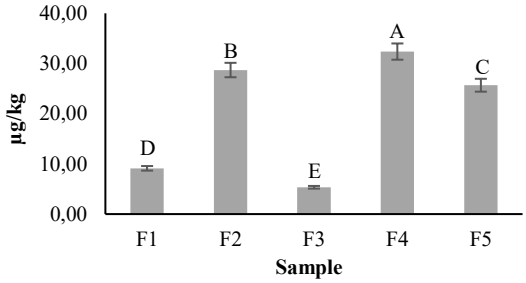

Figure 2 *Results of the total nitrosamines content (µg/kg) of the ripened sausages. Different letters indicate significant differences at p<0.05.*

3.3 Shelf-life prediction of ripened sausages with OLE during storage in MAP condition at 4°C

Kinetic models are a useful tool for the control and prediction of quality indices changes in foods. They are used to describe the formation of undesired compounds, aggregation in texture formation and inactivation of enzymes and microorganisms (Zhang *et al.,* 2021). This approach could be helpful to define the shelf-life of foods and thus the "best before" date to be reported on the label

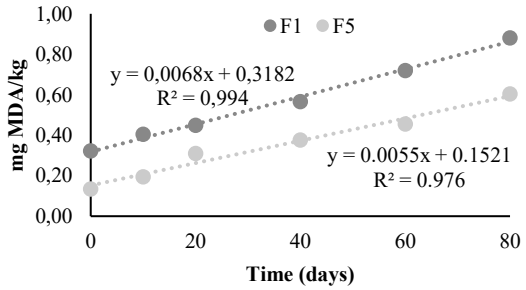

Figure 3 *Kinetic chart of F1 and F5 samples during MAP storage at 4°C.*

(Conte *et al.,* 2020). In meat products in which no contaminant microbial grown was observed (as in our samples) the evolution of the oxidative degradation is considered as target phenomena that prejudice the consumer acceptability of the products and so their shelf-life.
The first step concerned the identification of the most appropriate indicators leading to quality loss followed by the definition of the relevant acceptability limit. As regards ripened sausages, TBARs is considered one of the main analytical indices used to monitor the evolution of oxidation during storage and the acceptability limit is equal to 1 mg MDA/kg (Ockerman, 1976) that was reached in F4 samples after 70 days of storage (data not showed). In the next step, the reaction order of quality index was estimated based on R^2 obtained from MDA levels change of F1 (R^2=0.994) and F5 (R^2=0.976) as a function of the storage time (Figure 3). These results showed that the chose quality index fitted with the zero-order reaction model. Finally, data describing the evolution of the oxidative indicator as a function of time were submitted to modelling according to the equation (1) reported in Manzocco *et al.* (2016):

$$SL = \frac{I_{lim} - I_0}{k} \quad (1)$$

where SL is shelf life; I_{lim} is the oxidative value corresponding to the previously defined acceptability limit; I_0 is the value of the oxidative indicator just after sausages production; k is the rate constant. Based on the kinetic models obtained, the acceptability limit defined for lipid oxidation of the F1 and F5 samples will be reached, respectively, after 99 and 157 days of storage in MAP at 4 ºC.

4. Conclusions and Future Perspectives

To conclude this oral communication, even if is just an extract of the whole studies, showed the effectiveness of using the OLE for the reduction of nitrate and nitrite level in the ripened sausages through three different experimental steps. Together with an effective reduction of the nitrate/nitrite residual content, the replacement of the synthetic additives with the extract did not affect the hygiene and safety parameters of the sausages produced at laboratory scale. Based on these results, a *scale-up* approach was also experimented. In this case, the results of colour parameters, TBARs-test and total nitrosamines content showed the antioxidant effect of the extract. In addition, to predict the time needed to exceed the limit of level of incipient rancidity ripened sausages during storage, a shelf-life study was carried out. In this case, the kinetic model obtained confirm the effectiveness of using OLE as an alternative natural additive to improve and prolong the shelf-life of ripened pork sausages.

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Leveraging Lactic Acid Bacteria for new sustainable processes

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This Ph.D. thesis dealt with the phenotypic characterization of a microbial core (SMC) of Lactic Acid Bacteria (LAB) selected from the "University of Parma Culture Collection". The investigation of LAB functional potentialities aims to valorize the collection biodiversity and develop targeted fermentation processes for agri-food waste recovery. Results showed the aptitude of LAB to metabolize different compounds characterizing the composition of many agri-food waste and by-products, highlighting meanwhile their capability of growing far from the optimal conditions. Phenotyping microarrays represent a powerful approach to implement the genotypic knowledge on bacterial physiology and to move towards novel industrial applications of LAB.

Caratterizzazione funzionale di una collezione di batteri lattici per lo sviluppo di processi industriali sostenibili

Questa tesi di dottorato ha riguardato la caratterizzazione fenotipica di un core microbico (SMC) di batteri lattici (LAB) selezionati dalla collezione microbica di ateneo. Lo studio delle potenzialità funzionali dei LAB mira alla valorizzazione della biodiversità della collezione e allo sviluppo di processi specifici per il recupero di scarti agro-industriali. I risultati hanno mostrato l'attitudine dei LAB a metabolizzare sostanze caratterizzanti numerosi scarti agro-alimentari, evidenziando contemporaneamente la loro capacità di crescere in condizioni di stress. L'approccio fenotipico rappresenta un potente mezzo per implementare le conoscenze genotipiche sulla fisiologia batterica e per indirizzare la ricerca verso nuove applicazioni industriali dei LAB.

Keywords: phenotypic microarrays; biodiversity; lactic acid bacteria; waste recovery.

1. Introduction

In accordance with part of the Ph.D. dissertation project previously described (Troiani, 2021), this oral communication reports the core results of the following activities directed to:

A1) define the metabolic profile of the SMC's strains;
A2) study the microbial flexibility of growing under stressful environmental conditions;
A3) investigate the technological potentialities of strains concerning the bioplastic industry;
A4) predict the microbial behaviour for targeted fermentation processes.

2. Exploitation of microbial biodiversity

Microorganisms represent the greatest biodiversity in every ecosystem. The University of Parma owns a wide microbial collection, denominated "University of Parma Culture Collection" (UPCC), mainly composed of Lactic Acid Bacteria (LAB) isolated from diverse food matrices. LAB are widespread in nature, especially in food and human, and they are employed in many industrial applications (Buron-Moles *et al.*, 2019). Over the time, they evolved the ability to metabolize several carbon sources and tolerate various environmental conditions, thus becoming able to colonize many different habitats.

Current technologies in genome sequencing, bioinformatics, and high-throughput screening techniques applied to large microbial strain collections give us new opportunities in natural product discovery (Steele *et al.*, 2019), such as precursors of bioplastics, to move towards a sustainable economic system (Bosco *et al.*, 2021). The implementation of a circular economy model allows to recover waste and by-products produced every day all around the world and to reduce the over-exploitation of natural resources and environment (Venkata Mohan *et al.*, 2020), by providing significant economic benefits (Socas-Rodríguez *et al.*, 2021) and promoting sustainable development (Picot-Allain *et al.*, 2020). Producing interesting molecules for the industry by biological methods is one of the strategies of the circular economy, especially in the agri-food sector where food waste and by-products can be used as alternative fermentation matrices (Hadj Saadoun *et al.*, 2022).

The term 'phenotype' does not only mean the set of observable traits of an organism. Phenotyping aims to explore strain's niche-specific metabolic traits related to cell physiology and growth, such as substrate consumption, resistance to chemicals, and osmolyte tolerance or other stresses, thus establishing the potential adaptation under specific environmental conditions. Changes in phenotype depend on the interweaving interactions between environmental pressure and genotype (Acin-Albiac *et al.*, 2020). Studies of comparative genomics and metagenomics have been extensively employed to indagate the potential functionality of microbes,

while (meta)transcriptomics especially focused on mechanisms for niche adaptation. Metabolic traits interest the phenotype expression but, whilst data analysis methodologies and technologies for sequence-based omics quickly evolved, the systematic study of phenotype profiling stayed somehow hampered by some limitations (Houle *et al.*, 2010). Techniques like Biolog System make possible to screen many phenotypes concurrently thus introducing phenomics as the last of the omics techniques, whose implementation still has wide margins (Acin-Albiac *et al.*, 2020). However, experiments are expensive, time-consuming, and manually intensive to perform. Machine learning is a broad field of advanced computational and statistical methods whose models can help to describe complex data relationships and to facilitate the interpretation of large sets of data in all sectors, including microbiology. A combination of genotypic and phenotypic data provides an insight into the metabolic capabilities of industrially relevant strains, disclosing their potential application for the biotransformation of various substrates, including agro-industrial waste and by-products. Amplified Fragment Length Polymorphism (AFLP) is a well-known PCR-based technique that generates genomic fingerprints (Ramadan, 2022). This is largely shown by wet lab analyses, but no computational model is currently available to exploit its power.

3. Materials and Methods

The SMC consists of 150 strains of LAB of food origin belonging to the University of Parma Culture Collection (UPCC). The screening involved species belonging to 12 genera, previously isolated across a 10 years temporal range (2012-2022) from several food matrices, especially of dairy origin. Ecological diversity indices were used to describe the biodiversity of the microbial core (Table 1).

3.1 Genotyping

The DNA of each strain was extracted and used to perform the Amplified Fragment Lenght Polymorfism (AFLP) as reported by Bertani *et al.* (2019). Resulting electropherograms were analysed through Bionumerics Software v 8.0 (Applied Maths NV, Belgium) to have a clear fingerprint of the SMC.

3.2 Phenotyping

3.2.1 Metabolic activity on several carbon sources

Metabolic profiling of the SMC was evaluated through Biolog GENIII MicroPlates (BIOLOG Inc.©, USA) which incorporate a patented tetrazolium violet dye used as colorimetric indicator of the microbial activity on 71 different carbon sources. The final concentration of the cell suspension used for the inoculum ranged between 90-98% of transmittance. The microplates were incubated at the optimal temperature of strains for 72 h. The metabolic activity was detected by absorbance reading at 590nm at a single end-point (72 h). Raw data were referred against the negative control well and normalized by the Average Well Color Development (AWCD, average absorbance of all wells considered). Results were elaborated with Rstudio (R version 4.2.0).

Ecological diversity indices were adapted as reported in Table 1 to estimate also the metabolic functional diversity of the SMC (Daly *et al.*, 2018; Dubey *et al.*, 2022).

Table 1 *Ecological diversity indices used to describe the biodiversity of the SMC and its metabolic variability.*

Index	Formula	SMC biodiversity	Metabolic functional diversity
Richness (S) (SR)		Total number of species in the microbial community	Total number of substrates utilized by each strain (normalized value at 72h ≥ 1)
% SR	$\%\,SR = \left(\frac{SR}{n}\right) * 100$	/	Percentage of substrates assimilated
Shannon Diversity Index (H')	$H' = -\sum_{i=1}^{n} Pi * lnPi$	Entropy or disorder of the population $Pi = ni/N$ proportional abundance of species *i* where: *ni* = number of strains of species *i* *N* = total number of strains	Diversity of substrate utilization pattern where: $Pi = Rsi/\sum Rsi$ is the proportional color development of the well over the total color development of all wells of a plate, with *Rsi* = normalized OD value
Pielou's Evenness Index (E)	$E = \frac{H'}{logS} = H'/\ln(SR)$	Equitability of the population's species abundance distribution	Equitability of C source assimilation across all utilized substrates
Simpson Dominance Index (D)	$D = \sum_{i=1}^{n} Pi^2$	Probability that two strains randomly picked up from the community belong to the same species	/

Simpson Dominance Index (D')	$D' = 1/D$	Weight the common species more than the rare ones	/

Table 2 *Non-physiological conditions utilized to evaluate microbial environmental tolerance.*

Condition	Temperature (°C)	pH	NaCl (%)
Tmax	50	6.5	0
Tmin	15	6.5	0
pHmax	30-37	9	0
pHmin	30-37	4	0
salt	30-37	6.5	6

3.2.2 Tolerance to extreme growing conditions

The aptitude of strains to grow and colonize habitats particularly different from the optimal laboratory cultivation was investigated by phenotyping microarrays. Strains were inoculated (3% v/v) in 96-well plates on their optimal medium properly modified as reported in Table 2. Microplates were incubated until 72 h in aerobic condition. Growth was indirectly measured through absorbance readings at 590 nm, at 9 different time point (0h, 2h, 4h, 6h, 8h, 16h, 24h, 48h, and 72h) by using a BIOLOG MicroReader Station (BIOLOG Inc.©, USA). Results were referred against the blank and the variability of tolerating non-physiological conditions was described by the areas under the various sections of the curve: 0-8 h, 8-16 h, 16-24 h, 24-48 h, 48-72 h. Not to lose important information at the beginning of the growth, intervals of 2 h from 0 to 8 h were evaluated. Thanks to a collaboration with the Department of Mathematical, Physical and Computer Sciences, raw data were also elaborated with Phyton to reduce the dimensionality of the dataset through t-SNE analysis.

3.2.3 Technological capabilities

The capability of LAB to produce key-molecules for the bioplastic industry was investigated.

Lactic acid (LA) is the building block of polylactic acids (PLAs), widely used as a sustainable alternative to oil-based plastics. From 24 h old cultures grown in optimal conditions, the two isoforms of LA were determined by enzymatic method (Megazyme®, Ireland) measuring the OD at 340nm. Based on the highest amount and purity (> 97%) of D-LA produced, two strains (UPPC-4516 and UPCC-2214) were selected. During the period abroad at the University of Natural Resources and Life Science of Vienna, the cultures were scaled in 1L bioreactors (DASGIP AG, Jülich, Germany) carrying out a batch process with 20 g/L initial glucose, followed by a fed-batch one after total glucose consumption (constant feeding rate = 2,5 ml/h of 50 % (w/v) glucose). The process was then optimized by keeping constant the pH = 6.2 and increasing the feeding rate (4 ml/h for UPCC-2214 and 6 ml/h for UPPC-4516). The production of polyhydroxyalkanoates (PHAs), other precursors of bioplastics, was explored as suggested by Bosco *et al.* (2021) with some modifications to the protocol. Strains were firstly screened on Malt Extract Agar plates containing 0,5 µg/ml Nile Red in DSMO which binds to lipid molecules and emits fluorescence under UV light. Plates were incubated at the optimal temperature in the dark until 48 h. Strains that showed fluorescence were moved in liquid culture to boost the biomass production and extract as much lipids as possible to make their chemical characterization possible.

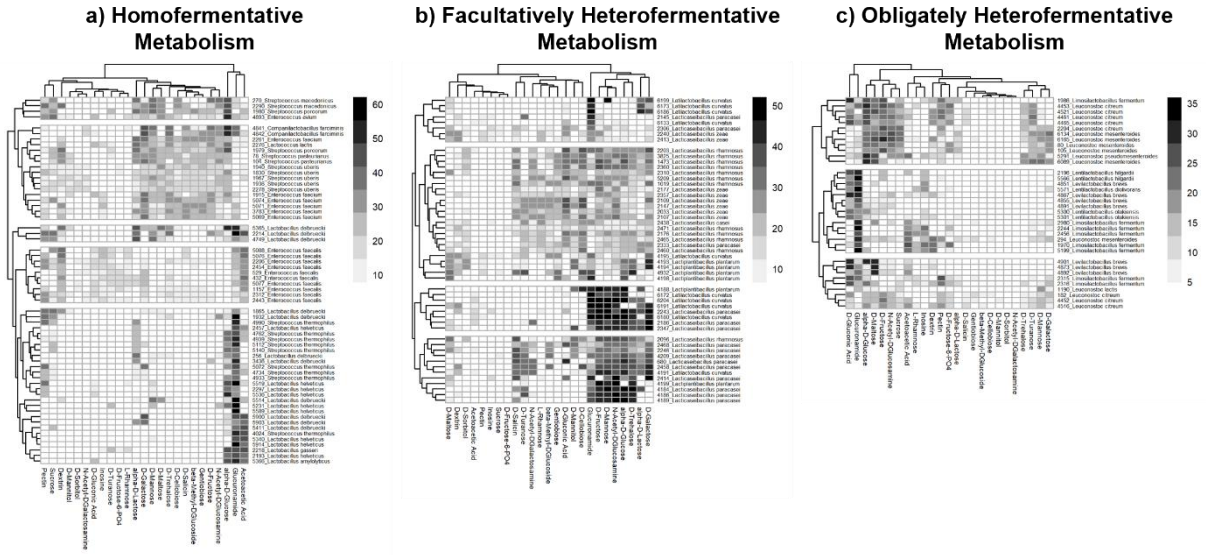
3.3 Machine learning for prediction

This part was focused on the exploitation of the fragment length distribution profile of bacterial genomes provided by the AFLP technology to obtain computational predictive models. Two different benchmark has been developed using genotypic and phenotypic information obtained from this work (141 strains) and extracted from publicly available database (in silico AFLP profiles and API-50 results from BacDive) (509 genomes). These datasets were used for training the machine learning models and classification algorithms to predict the metabolic traits encoded by bacteria according to their phylogenetic profiles were applied.

4. Results and Discussion

4.1 Biodiversity into the SMC

Indices calculated to describe the SMC's biodiversity gave us information concerning the entropy of the population (H′), the homogeneity of the community (E), and the dominance of some species over others (D'). The higher the number of species (S = 29), the higher H': H' = 1.32 indicates that the selection criteria guaranteed a good level of diversity into the SMC. E = 0.90 means a comparable distribution among all species as it ranges between 0 and 1. Despite the diversity, D' = 17.88 highlights that some species are more representative than others in terms of number of individuals. The AFLP clustering generally validated the biodiversity since it reflects the belonging to the species, as well as the metabolism characterizing microorganisms. Despite the overall evenness of clusters, the fingerprints of individual strains showed a certain level of intra-specific biodiversity and even more within the same genus, mainly due to the different source and moment of isolation. However, the distribution of metabolic profiles based on both the species and the metabolism type, was more disordered than the genotypic one indicating that the variability into a community is better represented by phenotyping than genotyping.



4.2 Phenotypic studies

4.2.1 Metabolic profiling

Figure 1 *Heatmaps of favourite C sources of the SMC's strains divided by type of microbial metabolism.*

The majority of LAB mostly metabolized 25 of the 71 carbon sources supplied. No strain showed any activity on gelatine, p-hydroxy-phenylacetic acid, γ-amino-butryric acid, and D-lactic acid methyl-ester. All other substrates were used by less than 30 strains over 150 tested. Ecological diversity indices were used also to express the variability among metabolic profiles. Phenotype of homofermentative bacteria (HMF, 62 strains), facultatively heterofermentative bacteria (FHTF, 51 strains), and obligately heterofermentative bacteria (OHTF, 37 strains) was evaluated separately (Figure 1). HMF showed a wider phenotypic variability than FHTF and OHTF as they belong to genera phylogenetically more divergent compared to the latter. Within HMF, it is remarked a metabolic heterogeneity inside the *Streptococcus* genus and within the species *L. delbruecki* and *L. helveticus.* Metabolic profiles of FHTF were homogeneous within each species except for *L. curvatus* whose strains showed different substrates assimilation capability, while, among OHTF, strains of *L. fermentum* showed different metabolic patterns. Among the favourite C sources of the screening there are monosaccharides, disaccharides, sugar derivatives and a purine nucleoside. Glucose was the most frequently used substrate, however most of other substrates can be actively consumed by LAB to levels comparable with it. Differently, some compounds were only metabolized by specific strains to a much higher measure than glucose. HMF and FHTF resulted to be more flexible and adaptable than OHTF. This versatility allows a wider spectrum of applications, while metabolically limited microorganisms can be involved in adaptive evolutionary studies by betting on their edges and addressing towards targeted selection processes for industrial specific uses.

4.2.2 Stress tolerance

The areas of the trapezes under the curve based on the different time intervals were used to describe the microbial growth and they were referred against the optimal condition. Only species composed of more than 5 individuals are here discussed. The further away the growth from the optimum and the longer the lag phase, the lower the slope of the curve and the lower the y-end. In the optimal condition, *Lacticaseibacillus* spp. and *L. plantarum* generally grew better than other species in terms of biomass, while *L. fermentum* was the fastest in the first 8 h. Applying an osmotic pressure, only *L. zeae* kept on growing until 72 h, although it reached less than half growth of the optimal one. *Lactobacillus* spp., *L. brevis* and *Streptococcus* spp. did not grow with 6% NaCl. Moving away from neutrality, *Lacticaseibacillus* spp. well tolerated both pH = 9 and pH = 4, with a remarkable preference for alkaline environments. However, *Lactobacillus* spp., *L. brevis,* and *L. fermentum* did not grow at pH = 9, as well as *Leu. mesenteroides* and *Streptococcus* spp. did not tolerate acidic environments. Finally, studying the growth at extreme temperatures, *L. rhamnosus* and *L. delbrueckii* well reacted to T = 50°C, with a short lag phase, contrarily to *L. curvatus*, *L. brevis*, *Leuconostoc* spp. and *Streptococcus* spp. At high temperature, all species generally reached the death phase before 72 h. On the other hand, growth at low temperature was characterized by a very long lag phase. This condition was well tolerated by *Lacticaseibacillus* spp., *L. plantarum,* and *L. curvatus*, but not by *L. fermentum*, *Streptococcus* spp. and *Lactobacillus* spp. Summarizing, in terms of adaptability, *Lacticaseibacillus* appears the most promising genus within the screening, while *Lactobacillus* spp., *Streptococcus* spp., and *L. brevis* struggle more than others to get out of their comfort zone. The t-SNE analysis clustered strains based on their similarities, confirming the behaviours cited above and highlighting differences among individuals belonging to the same species or genus.

4.2.3 Lactic acid production

Over 150 strains, 72 produced L-LA (> 80% of the total LA), 18 produced D-LA and the rest produced a racemic mixture of them. *L. delbrueckii* UPCC-2214 and *Leu. Citreum* UPCC-4516 resulted the main producers of D-LA with the highest isomeric purity. In small scale tests, they produced 20.40 g and 11.69 g in 72 h, respectively. They were scaled up in bioreactors and, after optimization, *Leu. Citreum* consumed the double amount of glucose (134.66 ± 14.21 g) compared to *L. delbrueckii* (71.77 ± 4.42 g) to finally produce about the same amount of lactate (58.33 ± 4.98 and 51.93 ± 1.22, respectively). In 58 h, *L. delbruecki* reached twice the yield (g/g) of *Leu. Citreum* (0.73 ± 0.03 and 0.43 ± 0.01, respectively), although presented a similar volumetric productivity (1.17 ± 0.03 and 1.02 ± 0.02, respectively). Cultivation in bioreactor allowed to further select UPCC-2214 as the most efficient strains of the SMC for the D-lactic acid production, and to increase the productivity.

4.2.4 PHA production

PHAs are produced and stored as lipidic granules inside the cell when the culture medium is strongly unbalanced for the C/N ratio. From the primary screening resulted 56 lipid-producing strains. This work is promising since the production of PHAs by LAB is still explorable, thus the next step is to chemically characterize the molecules produced and eventually scale the production up.

4.3 Prediction

As a result, we could correctly classify a good portion of phenotypic traits with an average accuracy of 0.8 and by using 70% of the data set for the training. Regarding the BacDive benchmark, all the computational models showed an accuracy greater than 0.6 (average 0.85) for every API-50 activity. Therefore, Random-Forest was the best machine-learning model for BacDive dataset. Similar results were obtained for the other benchmark with an average accuracy of 0.72. Feature importance techniques were also applied to produce a selection of fragment lengths associated with the predictive power within a given group of genomes and/or for a specific metabolic activity. On top of this selection, the idea is to perform functional enrichment of the selected fragments by comparing their position within the genome with the coordinates of genes for which functional annotation is already available.

5. Conclusions and Future Perspectives

The combination of genotypic and phenotypic data provides an insight into the functionalities of industrially relevant strains, displaying their potential leverage for the recovery of food waste and by-products and the production of added-value compounds, with the advantage of reducing costs and increasing sustainability. Results of this primary screening notice that LAB can metabolize substrates not traditionally associated with the matrices of isolation. Moreover, the variability on metabolic profiles and tolerance to stressful environmental conditions can allow us to better understand niche adaptations. Finally, besides the enrichment of the UPCC database, all data obtained from this study will be useful to develop an Artificial Intelligence (AI) algorithm to predict the microbial behaviour in terms of what LAB metabolize, where and how they grow, what they produce and in which conditions. The predictive approach will allow to design ad-hoc experimentation and have a preliminary idea of the feasibility and microbial performance to avoid waste of material, work, and time in the laboratory. The application of AI to investigate the biodiversity of microorganisms represents a powerful approach to expand the existing knowledge on bacterial physiology and can set a step in developing novel industrial applications of LAB.

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Dual Approaches to Investigate the Infant Food Microbiome and *Listeria monocytogenes* Behaviour under Severe Acidic Conditions

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The aim of this PhD thesis research project was to implement untargeted (metataxonomic analysis) and targeted (culturing and qPCR) approaches to decipher the distribution of pathogens and how they persist inside a complex microbial community of an infant food processing line through time and space. Furthermore, a comprehension of the behavioural mechanisms (phenotypes, gene expression) of a *Listeria monocytogenes* pathogenic strain after adaptation at low acidic conditions led to conclusions on possible predictive models for the benefit of food safety. This PhD is part of the European Project "SAFFI - Safe Food for Infants in the EU and China".

Doppio Approccio per Studiare il Microbioma degli Alimenti per Infanzia e il Comportamento di *Listeria monocytogenes* in Condizioni di Elevata Acidità

L'obiettivo di questo progetto di ricerca di tesi di dottorato è stato quello di implementare approcci non mirati (analisi metatassonomica) e mirati (colturali e qPCR) per definire la distribuzione dei patogeni e il modo in cui essi persistono all'interno di una complessa comunità microbica come quella di una linea di trasformazione di alimenti per l'infanzia, sia nel tempo che nello spazio. Inoltre, la comprensione del comportamento (i fenotipo, espressione genica) di un ceppo patogeno di *Listeria monocytogenes* dopo l'adattamento a condizioni di bassa acidità ha portato a conclusioni su possibili modelli predittivi a vantaggio della sicurezza alimentare. (SAFFI)

Keywords: infant food, pathogens, DNA, real-time PCR, metataxonomic analysis, in vitro, behaviour, severe acidity, adaptation, stress.

1. Introduction

In line with the previous year's manuscripts this oral communication reports the main results obtained following the activities directed to:

A1) detect targeted pathogens by combining culture dependent and culture independent methods.
A2) find out routes of transmission and determine the microbial taxonomic composition, focusing on its protagonist's associations (co - exclusion / co - existence), in other words of correlations between the most abundant taxa and the targeted pathogens.
A3) determine the microbial response of *Listeria monocytogenes* under acidic conditions by evaluating the robustness gained through a survival under severe acidity, through phenotype and transcriptomics.

All these aspects are relevant matters of great interest as foodborne pathogens and foodborne diseases, are still a significant public health issue worldwide and an immense challenge for food industries (World Health Organization, 2017, 2015; Zwirzitz et al., 2020).

2. Culture - dependent and Culture - independent Methods

Both approaches are directed by the same aim of detecting targeted pathogens. Culture - dependent approaches include the classical microbiological analysis and the analysis of whole - genome sequencing (WGS). Classical microbiological analysis consists by the traditional culture methods in generic and selective medium and under optimal incubation conditions, in order to let the time to the agent to grow and in position to be culturable. WGS is a molecular subtyping method eventually used in routine pathogen surveillance. Permits to evaluate and establish whether isolates derive from a same persistent clone inside different compartment along a food chain, as well as validates the causative agent related to a multi - country outbreak (Koutsoumanis et al., 2020, 2019; Kovac, 2019). Culture - independent approaches mainly refer to real - time PCR, 16S rRNA - gene amplicons sequencing (metataxonomics) and shotgun sequencing (metagenomics). Firstly, real - time PCR is used as a targeted method for the detection of pathogens in the whole DNA of a complex sample matrix (Kralik and Ricchi, 2017). Moreover, both metataxonomic analysis and metagenomics, as untargeted methods, are able to investigate uncultivable microorganisms inside the whole genetic content of communities. Amplicon sequencing is making possible to profile the succession of entire microbial populations over time at various taxonomic levels and have an overview on the taxonomy, composition and diversity of bacterial communities. Metagenomics offer insight into the metabolic pathways and the community genetic signatures (antimicrobial resistance genes, virulence genes) of a microbiome and can direct track the presence of unculturable organisms (even at the strain level) and as well predict potential functions encoded by the microbial communities (Jagadeesan et al., 2019).

3. *Listeria monocytogenes* and Acid Stress

Listeria monocytogenes is a foodborne pathogen, ubiquitously present thanks to its extreme adaptability under a wide range of environments and food - vehicles. It can easily pass from the saprophytic state to the invasive (intracellular) one, causing severe infections (listeriosis), mainly to immunocompromised members of the population (Buchanan et al., 2017; Freitag et al., 2009). *L. monocytogenes* is known to persist over long periods of time within food production facilities, colonising favourable niches. Furthermore, the processing environments to a large degree are characterised by severe conditions, like nutrients scarcity, acidic pH, high osmolarity, heat shock and ecological competitions that can impose various levels of stress resulting in lethal (irrevocable microbial cells damage) or sublethal (survival permission) stresses (Ferreira et al., 2014).
Low acidity is mainly characterising production lines that include products from fruit and vegetable derivatives (such as fruit purees and juices) but also a condition during the cleaning and sanitisation process, where acids are commonly used. Weak acids have been used as food preservatives and their mode of action is represented at Figure 1. Briefly weak organic acids can present in parallel their dissociated and undissociated state, depending on their acidic group state and the pH of the environment. The undissociated form is able to freely diffuse into the microbial cytoplasm till to reach an equilibrium (equal internal and external concentration). In this way the intracellular pH is reduced, the osmolarity of the cytoplasm is increased and general metabolic perturbations are observed (Hirshfield et al., 2003).

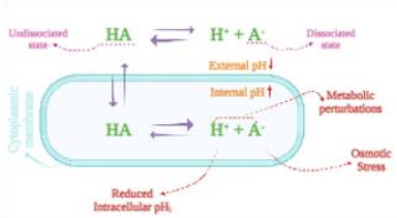

Figure 1 *Dissociation Equilibrium of Weak Organic Acids on the Cell.*

Listeria monocytogenes adaptation to sublethal conditions, increases its resistance that results in significant gene and protein expression profile changes mainly associated with the mobilisation of cellular mechanisms that deal with acids. *L. monocytogenes* responds to the acidic stress conditions through the production of various Acid Stress response Proteins (ASPs), such as proteins involved to the respiration (dehydrogenases (GuaB, PduQ, lmo0560) – reductases (YcgT)), osmolyte transport (GbuA), protein folding and repair (Chapronin, GroEL, ClpP), flagella synthesis (FlaA) and metabolism (Pfk, GalE) (Soni et al., 2011). Furthermore, *Lm* possesses various acid adaptive mechanisms including the Adaptive Acid Tolerance Response (ATR), the Glutamate Decarboxylase (GAD) system and the Arginine Deaminase (ADI) system that help the microorganism regulate the internal cytoplasmic pH and in general overcome the acidic environments (NicAogáin and O'Byrne, 2016).

4. Materials and Methods

From October 2021 to December 2022 a total number of two hundred and thirty - five samples were collected during various stages of an infant food process line and more in specific powdered cereal based infant food. Four types of samples were investigated: Raw Material (RM), Intermediate (IP) and Final (FP) Products, as well as Environmental swabs (ENV). Moreover, longitudinally the sampling period has been conducted in four campaigns: C01 (October 2021 - January 2022), C02 (January 2022 - June 2022), C03 (June 2022 - October 2022), C04 (October 2022 - December 2022). The presence of five targeted foodborne pathogens - *Listeria monocytogenes* (*Lm*), *Salmonella* spp. (*S*), *Staphylococcus aureus* (*Sa*), *Bacillus cereus* (*Bc*) and *Clostridium perfringens* (*Cp*) - was examined through traditional culturing methods prior and subsequent to twenty - four hours of generic enrichment, whereupon pathogens isolation took place in selective media. On each sample molecular techniques, with focus on the whole DNA, were followed. Specifically, 16S rRNA amplicon - based sequencing was performed, mainly investigating the Amplicon Sequence Variants (ASVs) distribution and in parallel real - time PCR used to enhance the detection of the targeted pathogens. What concerns the *Listeria monocytogenes* behaviour under acidic conditions, a single strain (10403S) was chosen as a well monitored case. A weak organic acid was used to reach the requested acidic conditions and that was citric acid. A preculture of *L. monocytogenes* was cultivated at Tryptone Soya Broth supplemented with Yeast Extract (TSBYE) till the stationary phase and then was inoculated at three theses: thesis 1 - control TSBYE at pH 7.2, thesis 2 - TSBYE at pH 5.5 and thesis 3 - TSBYE at pH 5.2. The three cultures let to adapt till twenty - four hours of growth under continuous agitation at thirty - seven degrees Celsius. The capacity of each of the thesis to adapt and increase acid resistance was measured at four time points during growth (t0, t6, t12, t24) by performing lethal acidic shock at pH 2 for thirty minutes. The capacity of the cultures to survive this shock was investigated phenotypically (counts) and at RNA expression level, by sequencing the whole transcriptome (RNA - seq).

5. Results and Discussion

5.1 Bacterial Communities Structure and Metataxonomic Composition

Considering as a factor the type of sample (RM, IP, FP, ENV) there is no clear segregation in between the microbial communities. If the focus is put upon time, then most of the microbiota variability is explained (PERMANOVA, P[FDR] < 0.001, R^2 = 0.4), making time the major driver of the biodiversity of the samples (Figure 2). The impact of time is also seen through the α - diversity measures, where a wave - like pattern is observed along the fourteen months of sampling.

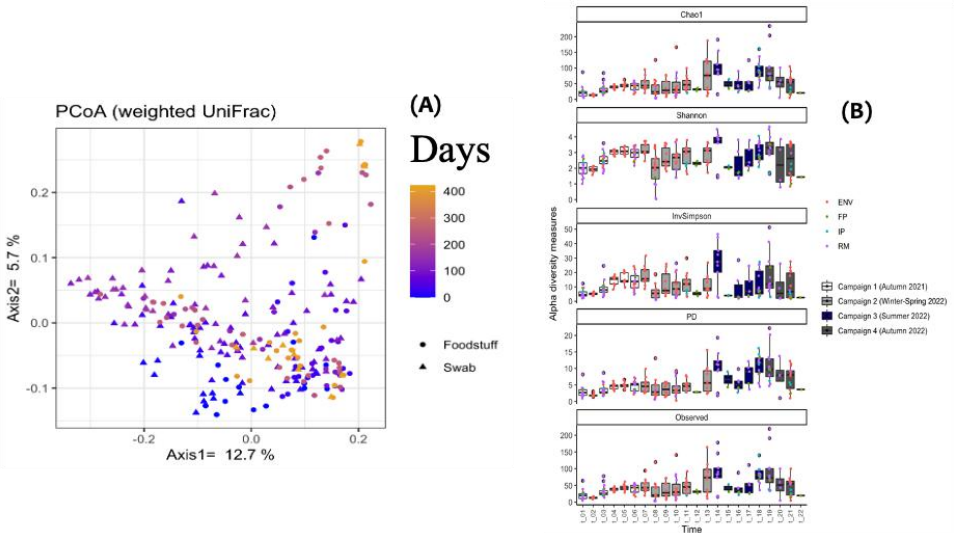

Figure 2 Alpha - diversity (B) and Beta - diversity (A) Measures of Bacterial Communities.
(A) PCoA plot based on weighted UniFrac β - diversity distance (PERMANOVA, R2 = 0.35, P[FDR] < 0.001). (B) Box plots of α - diversity metrics display the fourteen months of sampling in twenty-two blocks and four campaigns (Kruskal - Wallis, P < 0.001).

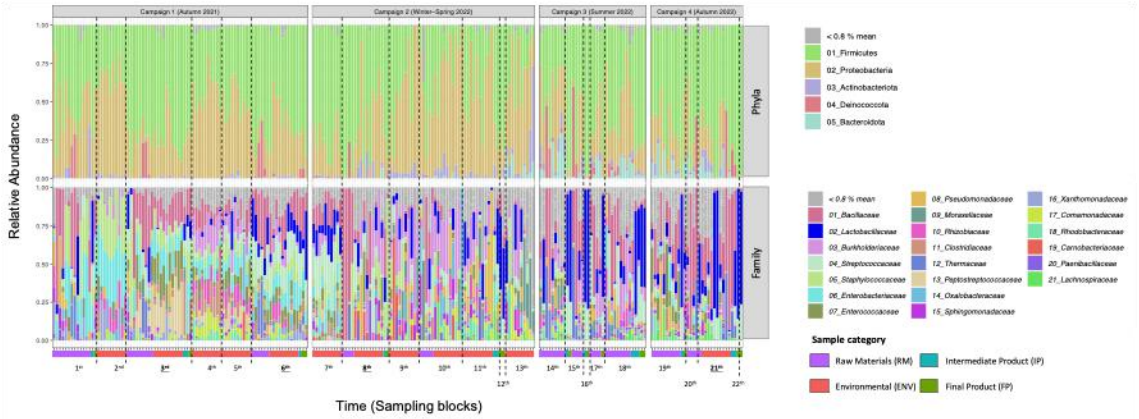

Figure 3 Overview of the Microbiota Composition at Phyla and Family Level.
Stacked bar plots representing the microbiota composition - relative abundance - in phylum and family taxa ranks with colour coding keys. The samples are grouped following the temporal sampling order and thus are divided by sampling blocks. The legends contain the taxa sorted from the most to the least abundant and only taxa >0.8 % of average abundances are displayed.

In Figure 3 is represented the microbiota distribution in relation to the sampling time, divided in sampling campaigns and subsequently by sample type. The phyla Firmicutes, Proteobacteria, Actinobacteriota, Deinococcota and Bacteroidota are predominant and ubiquitously distributed in all samples. Furthermore, there is an evident temporal succession of families during time and more in particular when comparing the microbial composition of the four campaigns. For instance, *Enterobacteriaceae* and *Staphylococcaceae* are predominant in the first campaign, while *Lactobacillaceae*, *Lachnospiraceae* and *Enterococcaceae* abundances tend to increase along time.

5.2 Shared Taxa

The taxa distribution both at family and genus / species level were investigated through Venn diagrams (Figure 4) in order to identify the core taxa shared between the sample types (RM, IP, FP, ENV). Within the fifty - six

core families it was possible to detect *Bacillaceae*, *Clostridiaceae*, *Enterobacteriaceae* and *Staphylococcaceae*, while *Listeriaceae* was shared between RM, IP and ENV but was not detected in the FP. At the highest taxonomic level, the distribution for the targeted pathogen species or belonging genus was confirmed, except for *Salmonella* spp. that was not detected among the *Enterobacteriaceae* family.

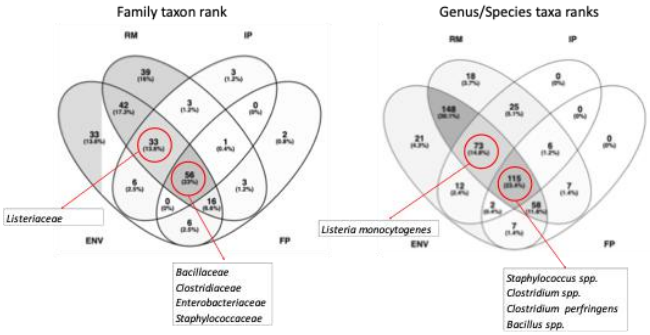

Figure 4 Venn Diagram - Shared Taxa.
Display of the number of shared taxa at the family and genu / species levels among the four sample types. Only taxa present in more than four samples are considered and the presence of families and genus / species of interest are highlighted.

5.3 Targeted Pathogens Detection

The two spore forming bacteria were the most present followed by *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella* spp. There are samples that contain more than one targeted pathogen. From their presence in the different sample types, it could be assumed that *Bacillus cereus* and *Clostridium perfringens* are introduced to the production through the RM and the other three pathogens through the ENV. From the networks (Figures 5 & 6) can be observed the straight correlation in presence between samples of the same production period but as well a certain degree of overlapping between the two targeted methods (qPCR in matrices and isolation).

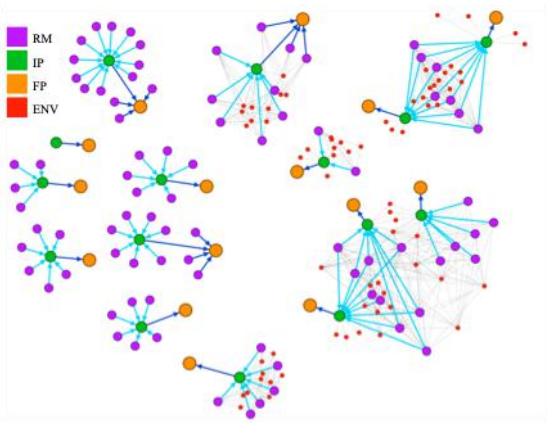

Figure 5 Network Displaying Links among Samples.
Nodes with different colours are representing the type of sample and edges are showing the connections and directions in between the samples in relation to the process

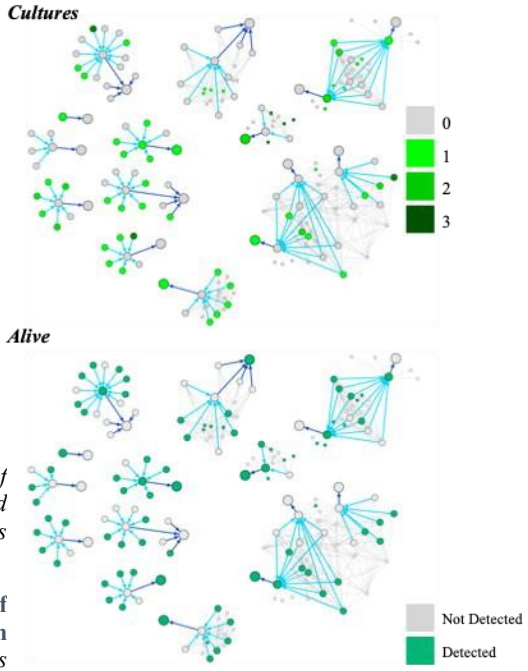

Figure 6 Detection of *Bacillus cereus* Correlating Results of Isolation (Cultures) and real - time PCR (Alive) in matrices. *The upper colour scale represents number of isolates and the lower colour scale detection or not in matrices*

5.4 *Listeria monocytogenes* Behaviour under Acidic Conditions

Listeria monocytogenes strain 10403S gained robustness after a long - term acid adaptation (LTAA) and more specifically the theses 2 (pH 5.5) and 3 (pH 5.2) showed significantly (T - test: P[FDR] < 0.001) higher resistance in comparison to the control (pH 7.2) when reaching the stationary phase, at twenty-four hours of growth. In the Figure 7 are represented the four points of interest, where the phenotypes and the response to the lethal shock were monitored. Moreover, observing the bar plots is clear the variation in robustness between the different phases of growth, as for example at hour six where the cells of all the theses are at the exponential

phase there is no resistance. From the twelfth hour of growth, it appears a slight resistance at the thesis 2 as is reaching the early stationary phase, while the thesis 3 is still at the exponential phase and in fact resistance was not presented. The data from RNA - seq (Figure 8) showed a difference in the GC content profile, where the treated (thesis 2 & 3) cases had higher GC content in comparison with the control at twelfth and twenty fourth hour.

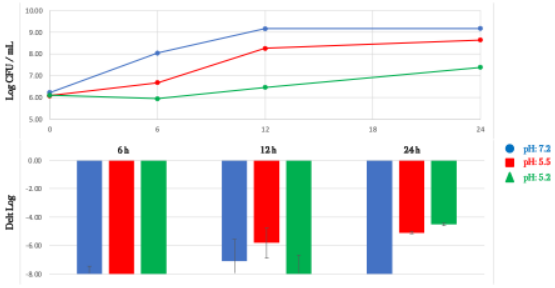

Figure 7 LTAA Model of *L. monocytogenes* 10403S Behaviour at the three Theses.
The four points of interest (t0, t6, t12, t24) that were further examined through RNA - seq are represented. The line plot is showing the dynamics of growth (logCFU/mL) and the bar plot the response to the lethal acid shock (Delta Log).

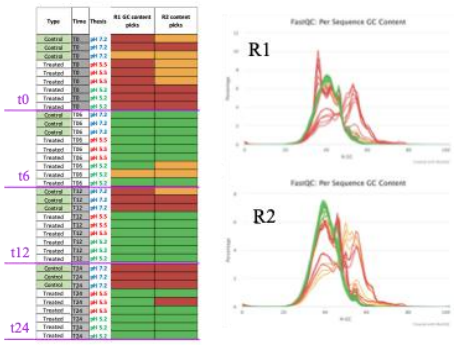

Figure 8 GC Content profiles of RNA - seq Raw Data (Illumina paired - end reads).
For the colour lines in the graph of GC content distribution (R1 &2) refer to the heatmap.

6. Conclusions and Future Perspectives

The infant food process line showed a high biodiversity through the time, given the nature of the microbiome present in such a habitat. The monitoring of both the environment and the foodstuff during the fourteen months of sampling made possible the more in - depth investigation of the routes of transmission of the targeted pathogens, shedding light on prevalence and correlation dynamics. Correlations between the different approaches showed the way in which these methods can be utilised in order to give accurate responses on given issues at the food industry and enhance the food safety aspects. Furthermore, the research on behavioural dynamics of a pathogen, made possible to understand factors and conditions that permit resistance. To that scope further research activities containing analytical techniques (volatile compounds - biomarkers) could give a more complete prospective of the metabolic pathways and a faster detection method. Of course, further investigations of stress conditions, given the complexity of the cell system, could lead to an updated hurdles perspective.

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