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Exploitation of the Functional Potential of Autochthonous Microorganisms from Fermented Foods

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List of abbreviations

ACE	Angiotensin Converting Enzyme
AckA	Acetate Kinase
ANOVA	Analysis Of Variance
BCFA	Branched-Chain Fatty Acids
BHI	Brain Heart Infusion
BP	Bioactive Peptide
BSA	Bovine Serum Albumin
CEP	Cell-Envelope Proteinase
CMP	Caseinomacropeptide
CS	Citrate Synthase
DH	Degree Of Hydrolysis
DHAP	Dihydroxyacetone Phosphate
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
E.	*Escherichia*
Eno	Enolase
Ent.	*Enterococcus*
Fba	Fructose-Bisphosphate Aldolase
FDAA	N-A-(2,4-Dinitro-5-Fluorophenyl)-L-Alanine Amide
FF	Fermented Food
FRAP	Ferric Reducing Antioxidant Power
G3P	Glyceraldehyde-3-Phosphate
Gap	Glyceraldehyde-3-Phosphate Dehydrogenase
GlcK	Glucokinase
Gnd	6-Phosphogluconate Dehydrogenase
GpmA	Phosphoglycerate Mutase
GRAS	Generally Recognized As Safe
KEGG KO	Kyoto Encyclopedia Of Genes And Genomes Ontology Level
LAB	Lactic Acid Bacteria
LacA	Galactose-6-Phosphate Isomerase Subunit A
LacB	Galactose-6-Phosphate Isomerase Subunit B
LacC	Tagatose-6-Phosphate Kinase
LacD	Tagatose-1,6-Bisphosphate Aldolase
LacG	Phospho-B-Galactosidase
L.	*Listeria*
Lb.	*Lactobacillus* and related genera
Lc.	*Lactococcus*
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
Ldh	Lactate Dehydrogenase
Leuc.	*Leuconostoc*
LFQ	Label Free Quantification
MRD	Maximum Recovery Diluent
MRS	De Man Rogosa Sharpe
MS	Mass Spectrometry
MW	Molecular Weight
NCD	Non-Communicable Disease

NSLAB	Non-Starter Lactic Acid Bacteria
OD	Optical Density
OPA	O-Phthaldialdehyde
Opp	Oligopeptide Permease System
P.	*Pediococcus*
P	Phosphate
PBS	Phosphate-Buffered Saline
PCA	Principal Component Analysis
PC1	Principal Component 1
PC2	Principal Component 2
Pdh	Pyruvate Dehydrogenase
Pfk	Phosphofructokinase
Pgi	Glucose-6-Phosphate Isomerase
Pgk	Phosphoglycerate Kinase
Pgl	6-Phosphogluconolactonase
PH	Extent Of Protein Hydrolysis
Pk	Pyruvate Kinase
PM	Pea Medium
PPC	Pea Protein Concentrate
Ps.	*Pseudomonas*
Pta	Phosphotransacetylase
PTS-G	Glucose-Specific Phosphotransferase System
PTS-Lac	Lactose-Specific Phosphotransferase System
Pyc	Pyruvate Carboxylase
QPS	Qualified Presumption of Safety
RAPD	Random Amplification of Polymorphic DNA
Salm.	*Salmonella*
SCFA	Short-Chain Fatty Acids
SE-HPLC	Size Exclusion High Performance Liquid Chromatography
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SLAB	Starter Lactic Acid Bacteria
SSF	Simulated Salivary Fluid
St.	*Streptococcus*
Staph.	*Staphylococcus*
TBE	Tris/Borate/EDTA Buffer
TFA	Trifluoroacetic Acid
Tpi	Triose-Phosphate Isomerase
TPTZ	2,4,6-Tris(2-Pyridyl)-S-Triazine
W.	*Weisella*
WM	Whey Medium
WPC	Whey Protein Concentrate
WPM	Whey-Pea Medium
Xfp	Xylulose-5-Phosphate Phosphoketolase
Zwf	Glucose-6-Phosphate Dehydrogenase
α-La	α-lactalbumin
β-Lg	β-lactoglobulin

Preface

The global food system is currently facing major challenges from both a health and environmental perspective. On one side, non-communicable diseases (NCDs) such as obesity, diabetes, and cardiovascular disorders continue to rise, calling for dietary strategies that can actively support human health. On the other hand, the intensive production of animal-based proteins has a significant environmental impact, which is driving a growing interest in plant-based alternatives. At the same time, large amounts of nutrient-rich by-products from the animal industry remain underutilized. These issues highlight the urgent need to develop more sustainable and circular approaches to food production, capable of valorizing available resources while promoting human well-being.

Within this context, fermentation has re-emerged as a valuable and versatile approach to address both health and sustainability challenges. Traditionally used to preserve food and improve sensory properties and shelf life, fermentation is now recognized as a natural biotechnological process that can enhance nutritional value and generate bioactive compounds beneficial for human health. Recently, there has been a growing interest in fermentations carried out with autochthonous microorganisms. This trend reverses the impact of industrialization, which replaced natural microbial consortia with standardized starter cultures, thereby reducing the microbial diversity and functional potential of fermented foods (FFs). Among the microorganisms involved in food fermentation, lactic acid bacteria (LAB) play a leading role due to their safety and adaptability features. Their metabolic activity (e.g., proteolysis) can lead to the formation of a wide range of compounds with potential health-promoting properties, contributing to the nutritional and functional value of resulting food products.

Based on these premises, this PhD project aimed to explore the potential of autochthonous LAB to generate health-promoting properties from food proteins through fermentation. To represent different protein sources, whey proteins were used as a model for animal-based substrates and pea proteins as a model for plant-based ones. Overall, this work highlights fermentation as a sustainable and health-oriented strategy to transform protein sources into health-promoting FFs, combining traditional microbial biodiversity with modern nutritional and environmental goals.

Summary

Protein valorization represents a significant challenge in the transition toward more sustainable and health-oriented food systems. Fermentation by autochthonous lactic acid bacteria (LAB) offers a promising approach to enhance the nutritional and functional potential of animal and plant proteins. Through their metabolic activity, LAB can drive the biotransformation of proteins into health-promoting compounds, while contributing to the sustainable use of underexploited protein sources. Based on this rationale, this PhD project explored the potential of autochthonous LAB to generate health-promoting properties from food proteins through fermentation. Two protein models were studied, representing animal (whey) and plant (pea) sources.

After isolating and identifying wild LAB strains from dairy and plant-based fermented foods (FFs), dairy isolates were screened for their ability to hydrolyze whey proteins and release low-molecular-weight peptides, which are most frequently reported to exert bioactive properties. In contrast, plant-derived isolates were tested on pea proteins.

The selected strains, twelve from dairy and seven from plant-based FFs, were used in fermentation trials in whey and pea protein media. The composition of the resulting microbial fermentates was characterized, and their biologically active properties were evaluated. Across all experimental conditions, LAB strains exhibited a strain-dependent behavior in terms of proteolytic activity and functional outcomes. Several strains were able to generate fermentates displaying antioxidant, ACE-inhibitory, and antimicrobial properties. Moreover, some fermentates modulated intestinal microbial communities, promoting beneficial taxa and supporting metabolic functions associated with gut health.

A promising strain, *Lacticaseibacillus paracasei* AF43, was selected for in-depth analysis and was used to ferment media containing whey protein, pea protein, or a mixture of both. Integrated proteomics, metabolite profiling, and functional assays revealed that the growth medium strongly shaped the metabolism of the strain and the bioactivity of the fermentates. Depending on the substrate, differences were observed in carbohydrate metabolism, with the strain shifting between homo- and heterofermentative routes. These adaptations affected growth efficiency and were accompanied by variations in metabolite utilization and accumulation, ultimately influencing the functional output of the fermentates. Additionally, the strain's tolerance to digestive transit was found to be markedly affected by the cultivation environment preceding gastrointestinal exposure, suggesting that specific nutritional contexts can pre-adapt LAB for enhanced survival during gastrointestinal transit. Together, these findings contribute to a deeper understanding of the interplay between microbial metabolism, protein substrates, and health-related functionalities, offering perspectives for the sustainable valorization of protein sources.

Chapter 1:
Introduction and Thesis Outline

1.1 Fermented Foods: from Tradition to Functional Nutrition

Fermented foods and beverages (FFs) have been an integral part of the human diet since the earliest stages of civilization, spanning all continents and diverse cultural contexts. Fermentation represents one of the oldest and most significant biochemical practices, with a history closely tied to an evolving understanding of microorganisms that, for millennia, remained unseen yet profoundly influenced food, health, and society (Baumgarthuber, 2021). While modern microbiology recognizes many bacteria, yeasts, and molds as beneficial, historical perceptions oscillated between trust in traditional fermentation methods and fear of microbial contamination. In 2021, the International Scientific Association for Probiotics and Prebiotics defined FFs as *foods made through desired microbial growth and enzymatic conversions of food components* (Marco et al., 2021). This definition highlights the crucial role of microbial activity in producing FFs, clearly distinguishing them from spoiled foods, while remaining broad enough to encompass the full spectrum of fermentation processes.

FFs can be classified based on the main agents of fermentation and the primary metabolites they produce (Marco et al., 2017). Alkaline fermentation involves the proteolytic breakdown of proteins, releasing ammonia and increasing pH. This process is typical of certain traditional African and Asian FFs, such as fermented soybeans and alkaline-fermented fish. It is primarily driven by *Bacillus* spp. and other proteolytic microorganisms, resulting in distinct flavors and textures (Owusu-Kwarteng et al., 2022). Acid fermentation is a process in which organic acids are the primary end products. The most common type is lactic fermentation, carried out mainly by lactic acid bacteria (LAB), in which sugars are converted into lactic acid, responsible for acidifying the food matrix, which contributes to both preservation and flavor development. LAB are primarily accountable for lactic acid production in a wide range of plant- and animal-based FFs. In contrast, *Propionibacterium* species produce propionic acid in products such as Swiss-type cheeses, adding distinctive flavor and contributing to preservation (Valentino et al., 2024). Yeasts are responsible for alcoholic fermentation, which produces ethanol and carbon dioxide, fundamental in the fermentation of beverages and leavened products (Buglass, 2011). In some contexts, alcoholic fermentation is followed by acetic fermentation driven by *Acetobacter*, in which ethanol is oxidized into acetic acid, desirable for vinegar and kombucha production, but undesirable in beer and wine making (Gomes et al., 2018).

Over time, fermentation has been applied to a remarkable diversity of food matrices, including dairy products, vegetables, cereals, legumes, meat, fish, and fruits. More than 5,000 distinct FFs are estimated to exist worldwide, contributing between 5-40 % of the human diet depending on the region (Li et al., 2022b). First fermentations were almost certainly accidental. Sugary substrates such as fruit pulp, honey water, and cereals spontaneously transformed under the influence of wild

yeasts, producing alcohol and organic acids. Over generations, humans learned to control these transformations, developing stable starter cultures that could be maintained and passed through generations (Baumgarthuber, 2021). In regions where animal farming was widespread, such as the Middle East, Europe, and India, the ready availability of milk from cows, goats, and sheep led to the development of fermented dairy products; as a result, fermented milk, cheese, and yoghurt became dietary cornerstones. In contrast, in East Asian societies, like China, Japan, and Korea, animal agriculture was limited. Consequently, their fermentation traditions centered on plant-based substrates, focusing on rice, soybeans, grains, vegetables, and fish. Meanwhile, across Africa, indigenous cereal grains like millet, sorghum, maize, and wheat are the milestones of FFs. Root crops such as cassava also play a vital role, since they are an essential part of their traditional diets (Tamang et al., 2020). This variety of products not only reflects regional food preferences and availability but also demonstrates the versatile application of this process (Hernández-Velázquez et al., 2024).

Traditionally, fermentation was valued for extending shelf life and enhancing the safety of food products, as microbial metabolism end-products, such as organic acids, ethanol, and bacteriocins, create a hostile environment for microbial spoilers and pathogens (Rezac et al., 2018). These metabolic outputs contribute significantly to the safety and microbiological stability of FFs. Fermentation may also improve the sensory properties of foods by developing desirable tastes, flavors, and textures not present in the raw substrate. Thanks to the action of enzymes - like proteases, amylases, and lipases - microbe metabolism breaks down food constituents, generating different compounds which enhance flavor and texture (Ray & Joshi, 2014). All these aspects have contributed to the worldwide relevance of FFs (Figure 1.1).

The industrial era radically altered fermentation's path: the late 19th-century hygiene movement, arising from germ theory's dissemination, reframed microbes primarily as pathogens. Industrial-produced FFs were marketed as cleaner and safer than homemade equivalents, often through advertising that portrayed traditional methods as unhygienic. Despite this, artisanal fermentation persisted in rural and domestic contexts. The late 20th and early 21st centuries have seen a resurgence in home fermentation, reversing the early 20th-century narrative that associated microbial presence with danger (Baumgarthuber, 2021).

Lately, FFs have experienced renewed popularity, with a market of about USD 15.8 billion in 2025 in Europe, projected to grow to USD 28.2 billion by 2035 (Future Market Insights, 2025). This growth is not only due to their preservative, technological, and sensory qualities, but also to a growing recognition of their health-promoting potential (Mukherjee et al., 2022). Recently, consumers' attention has focused on dietary strategies that support health, reflecting the ever more affirmed idea that food is medicine, not only sustenance or pleasure (Chugh & Kamal-Eldin, 2020). This shift in perspective is particularly urgent in the light of the global problem of non-

communicable diseases (NCDs): slow-developing and long-lasting diseases, such as cancer, diabetes, cardiovascular, respiratory, and neurodegenerative diseases, responsible for over 75 % of annual deaths worldwide (WHO, 2024).

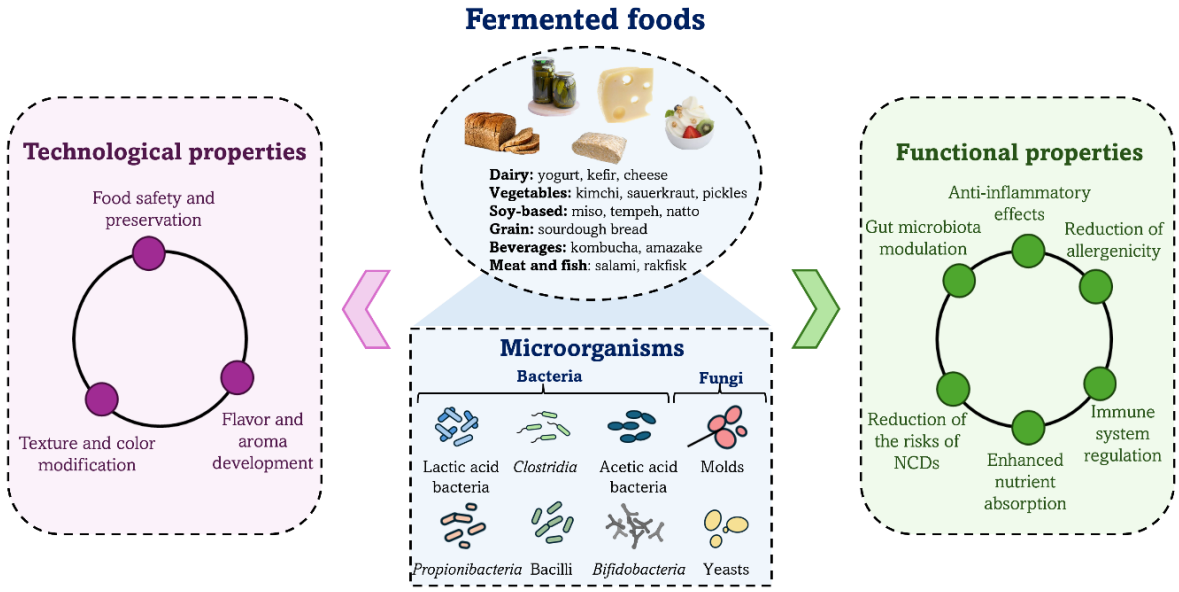

Figure 1.1 Schematic overview of the microorganisms involved in FFs and their associated technological and health-promoting properties

Evidence from epidemiological and clinical studies has underlined the role of FFs in the reduction of the risks related to these pathologies. For instance, the intake of FFs has been associated with a decrease in the risk of cardiovascular diseases, type 2 diabetes, obesity, and cancer (Cunningham et al., 2021).

The health benefits of FFs can be attributed primarily to the presence of live fermentation-related microorganisms and the functional compounds generated by microbial action, along with the removal of undesirable components during processing (Mukherjee et al., 2024). The microorganisms involved in fermentation carry out enzymatic transformations that generate bioactive peptides (BPs), organic acids, and exopolysaccharides, and enrich foods with essential nutrients such as vitamins, minerals, and amino acids. Many of these compounds have been linked to beneficial effects in gastrointestinal health, supported by human studies or inferred from *in vitro* and animal models (Abedin et al., 2024). For instance, recent research into the microbiota-gut-brain axis suggests that FFs may positively affect neurological health, including mood and cognitive function, through microbial metabolites such as γ-aminobutyric acid (Hou et al., 2024). Bioactive metabolites from fermentation can also exert immunomodulatory effects, thus improving host defense mechanisms and mitigating inflammatory responses (Zou et al., 2024). Additionally, microbial metabolism during fermentation can lead to the removal or substantial reduction of harmful compounds such as mycotoxins, lactose, vicine, covicine, trypsin inhibitors, phytic acid, and gluten,

thereby improving food quality, safety, and digestibility while minimizing potential adverse health effects (Chugh & Kamal-Eldin, 2020).

The health benefits of FFs also arise from their role as carriers of live microorganisms, sometimes including probiotics, to the gastrointestinal tract. Probiotics are *live microorganisms that, when administered in adequate amounts, confer a health benefit on the host* and must be isolated, strain-characterized, and supported by scientific evidence (Hill et al., 2014). While certain FFs are produced with proven probiotic starter cultures, many others contain a diverse range of bacteria, yeasts, and fungi that are not characterized to the strain level and thus do not meet the strict probiotic definition. Nonetheless, these FF-associated microorganisms may influence gut health directly, alongside the bioactive compounds generated during fermentation, and they represent a potential reservoir of future probiotic strains, particularly among LAB (Mukherjee et al., 2024). Some FFs are consumed with these live microorganisms intact, whereas others undergo pasteurization or filtration before consumption, eliminating viable microbes but preserving their metabolic products. The International Dairy Federation recommended that probiotic dairy FFs should contain at least 10^6-10^7 cfu/mL of probiotic microorganisms at the time of consumption to guarantee the corresponding beneficial effects (Halim et al., 2017). In contrast, probiotic non-dairy FFs are recommended to contain between 10^4 and 10^{10} cfu/mL of probiotics, depending on the product (Grujović et al., 2022). By competing with pathogens, preventing dysbiosis, and supporting beneficial gut bacteria, probiotics contribute to gastrointestinal and overall health (Chandrasekaran et al., 2024). These represent only some of the many health benefits attributed to FFs, which continue to be the subject of extensive scientific research.

In modern industrial food production, fermentation is carried out using standardized and well-characterized starter cultures, carefully chosen to ensure safety and regular product quality. These starters are usually selected for their technological traits such as acidification rate, texture, flavor improvement, and compatibility with large-scale production processes (Bintsis, 2018a). However, such controlled fermentations have reduced the diversity of FFs' indigenous microflora, limiting health-promoting features. Such loss of microbial diversity aligns with the *old friends* hypothesis, which suggests that reduced exposure to beneficial microorganisms, due in part to modern food production and a shift from natural FFs to standardized fermentation, has contributed to rising rates of allergic and autoimmune diseases (Baumgarthuber, 2021).

Traditional FFs may help restore the lost microbial contact, supporting immune system development and resilience (Mukherjee et al., 2024). In naturally fermented products, such as raw-milk cheeses, kimchi, sauerkraut, and artisanal meat products, fermentation is carried out spontaneously by the microflora naturally present in the raw materials without the addition of defined starter cultures (Beena Divya et al., 2012). These autochthonous microbial communities include microorganisms that can offer traits beneficial to prevent NCDs. Many authors have highlighted the

importance of naturally fermented products as a valuable source of strains with health-promoting properties, central to the development of functional foods as part of a health-supportive diet (Marco et al., 2017).

1.2 Lactic Acid Bacteria

Among the diverse microbial agents involved in food fermentation, LAB are acknowledged as central players due to their safety profile, acidification capacity, and ability to contribute desirable sensory and functional attributes of FFs. LAB are Gram-positive, catalase-negative, acid-tolerant, non-sporulating, and generally non-motile microorganisms that are facultative anaerobes with rod or cocci morphology (Bintsis, 2018). This group comprises several genera, including *Lactobacillus* and new genera (Zheng et al., 2020), *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Pediococcus*, *Enterococcus*, *Oenococcus*, *Weissella*, and *Carnobacterium*. They grow in a range of environments and can be mesophilic or thermophilic, with optimal growth temperatures between 25-45 °C and an optimal pH ranging from 5.5 to 6.2 (Ter et al., 2024). They are generally recognized as GRAS (Generally Recognized as Safe) by the Food and Drug Administration and, most of them, carry QPS (Qualified Presumption of Safety) status by the European Food Safety Authority, making them pre-assessed for use in food applications regarding human, animal, and environmental safety, except for some genera (e.g., *Enteroccoccus*) (Bintis, 2018; Cunningham et al., 2021). In addition to being the leading players in food fermentation, LAB are among the most used probiotics due to their ability to survive and adapt to various conditions within the gastrointestinal tract. As natural inhabitants of the human gut, they also play a key role in maintaining digestive health and supporting overall well-being (Shokryazdan et al., 2014; Suvorov, 2020).

LAB species are classified as nutrient-demanding bacteria due to their complex nutritional needs and limited ability to synthesize essential compounds such as amino acids, nucleotides, and vitamins (Hossain, 2022). To meet these requirements, LAB rely on their ability to break down the biochemical components of raw materials, such as lipids, proteins, and carbohydrates, during fermentation. Their metabolic activity not only supports bacterial growth but also enhances the digestibility, flavor, and nutritional and health-promoting value of the final product (Tamang et al., 2015). These transformations occur through three main metabolic pathways involved in lactic acid fermentation: glycolysis, lipolysis, and proteolysis, each contributing differently to microbial growth and substrate transformation (Grujović et al., 2022).

Among these processes, carbohydrate metabolism represents the primary energy-yielding pathway in LAB and is central to lactic acid fermentation. Based on the metabolic pathways employed, LAB are classified as homofermentative, obligately heterofermentative, or facultatively heterofermentative. Homofermentative LAB metabolize hexoses mainly via the Embden-Meyerhof-Parnas pathway, converting glucose almost exclusively into lactic acid, with the concomitant regeneration

of NAD^+ from NADH to maintain redox balance under anaerobic conditions (Gänzle, 2015). In contrast, obligately heterofermentative LAB utilize the phosphoketolase pathway, resulting in the production of lactic acid together with other metabolites such as ethanol or acetic acid and carbon dioxide. The relative proportions of these end products depend on substrate availability and environmental conditions, with acetic acid formation generally associated with higher energy yields (Bintsis, 2018b). Facultatively heterofermentative LAB occupy an intermediate metabolic strategy: these species (e.g., *Lactiplantibacillus plantarum*, *Lacticaseibacillus casei*) primarily ferment hexoses via glycolysis under conditions of abundant glucose, yielding mainly lactic acid, but can induce the phosphoketolase pathway when glucose availability is low or when pentoses are present, resulting in mixed end products including acetate and ethanol (Bintsis, 2018b; Gänzle, 2015). In addition to carbon source availability, environmental stress conditions such as low pH, oxidative stress, or nutrient limitation can also modulate the metabolic behavior of facultatively heterofermentative LAB. Under these conditions, a shift towards mixed-acid fermentation and increased acetate production may occur, enhancing ATP yield and contributing to stress tolerance and cellular homeostasis (Papadimitriou et al., 2016). This metabolic flexibility allows them to optimize adaptability in different ecological niches and fermentation environments. Lipolysis involves the hydrolysis of lipids into free fatty acids and glycerol, which can serve as precursors for flavor compounds or, to a lesser extent, as energy sources. However, the contribution of LAB to lipolysis is generally limited (Ter et al., 2024). On the contrary, proteolysis - the breakdown of complex proteins into smaller peptides and free amino acids - is a critical metabolic pathway in LAB, particularly in substrates rich in proteins but poor in amino acids, such as milk or some plant-based matrices (e.g., pulses) (Gorissen et al., 2018).

1.2.1 Protein Hydrolysis by LAB

Protein hydrolysis is a crucial process in fermentation, given that LAB are typically auxotrophic for several amino acids and thus rely heavily on external protein sources for nitrogen supply (Raveschot et al., 2020). Through their proteolytic system, LAB species hydrolyze proteins into smaller peptides and free amino acids (Figure 1.2). The proteolytic process begins extracellularly with cell-envelope proteinases (CEPs), which hydrolyze proteins into oligopeptides. These peptides, typically ranging from 4 to 30 amino acids in length, are then internalized via peptide transport systems, including the oligopeptide transporters (Opp system) that import peptides from 4 to 35 residues into the cell and the di-tripeptide transport system (Dpp and Dtp systems). Once inside the cytoplasm, various intracellular peptidases further degrade the peptides into smaller fragments - dipeptides, tripeptides, and free amino acids - that can be used for microbial metabolism, growth, and biosynthetic pathways (Picon et al., 2010).

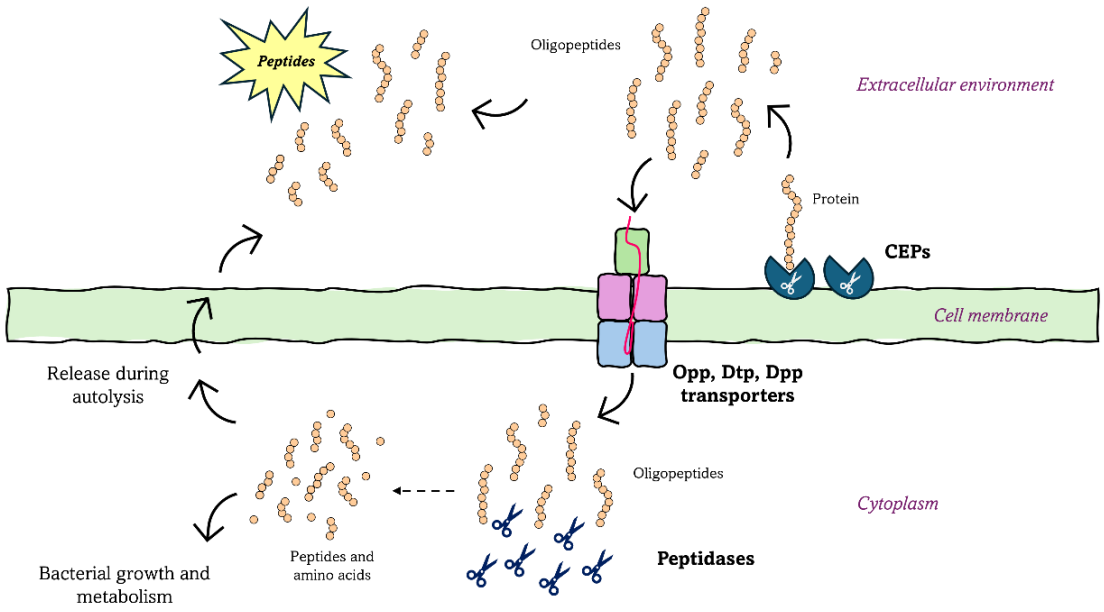

Figure 1.2 Schematic representation of protein hydrolysis by the proteolytic system of LAB

The significance of protein hydrolysis by LAB extends beyond microbial nutrition since the peptides and amino acids generated during fermentation serve important functionalities in food systems (Hajfathalian et al., 2018). As illustrated in Figure 1.2, oligopeptides released during LAB fermentation can accumulate extracellularly when not efficiently internalized by bacterial transport systems (Raveschot et al., 2018). Additionally, the autolysis of bacterial cells in later fermentation stages contributes further to the pool of low-molecular-weight peptides and free amino acids in the medium (Savijoki et al., 2006). This natural enrichment process enhances the nutritional and functional value of the FFs, contributing to improved digestibility, reduced allergenic potential of complex proteins, and enhanced health-supporting properties (Wu et al., 2025). As a matter of fact, the extracellular peptides, not utilized by LAB for nitrogen assimilation, can exhibit specific health-promoting effects, thus acting as BPs. BPs are short aminoacidic sequences (typically fewer than 50 amino acid residues) capable of exerting specific biological functions in the human body (Akbarian et al., 2022). A wide range of biological activities, including antihypertensive, cholesterol-lowering, anticancer, immunomodulatory, antimicrobial, antioxidant, and anti-inflammatory effects, are expressed by these protein fragments but are absent in the native protein and must be released through hydrolysis to exhibit their physiological benefits in humans (Cicero et al., 2017). Notably, protein hydrolysates rich in peptides with a low molecular weight (<3 kDa) appear to be the most effective in terms of bioactivity (Pimenta & De Lima, 2005).

1.2.2 Benefits of Microbial Protein Hydrolysis

Protein hydrolysates can be obtained with various methods, including chemical or enzymatic hydrolysis. Chemical hydrolysis involves the breakdown of proteins into peptides and amino acids using strong acids or bases under controlled conditions (Szopa et al., 2023). Enzymatic hydrolysis

employs specific proteases to selectively cleave peptide bonds in proteins, yielding hydrolysates with well-defined compositions and high efficiency within relatively short timeframes (Di Filippo et al., 2024). While effective, both approaches rely on purified enzymes or harsh chemicals, which may raise concerns regarding cost, food safety, and environmental impact. Alternatively, proteolysis through LAB fermentation represents a safe, cost-effective, and environmentally sustainable strategy for the functionalization of both animal- and plant-derived protein substrates (Nasri et al., 2022; Solieri et al., 2022).

In contrast to exogenous enzymatic or chemical hydrolysis, LAB-driven fermentation enables *in situ* bio-enhancement, whereby functional molecules are generated directly within the food matrix as a result of microbial metabolism. Additionally, LAB fermentation produces a diverse array of peptides, shaped by strain-specific proteolytic systems and diverse process parameters, rather than yielding a single, isolated compound. These peptides may act individually, additively, or synergistically, contributing to a broad bioactive profile (Raveschot et al., 2018). In addition to peptide production, LAB-driven fermentation can further enrich the product with a mixture of health-promoting metabolites, such as organic acids, bacteriocins, vitamins, and other compounds, which collectively may support human health and boost the effects of the protein-derived peptides (Raveschot et al., 2018). Importantly, these metabolites come not only from protein breakdown, but also from other microbial activities that occur during fermentation (Marco et al., 2017). Based on these considerations, the final product is not merely a protein hydrolysate, but rather a complex, protein-based FF enriched with peptides and microbial metabolites that together enhance its nutritional, functional, and health-promoting properties.

1.2.3 Optimization of Microbial Protein Hydrolysis

The success of LAB fermentation in enhancing protein functionality depends on the careful selection and optimization of strains and fermentation conditions to ensure efficient peptide release and improved biological properties (Figure 1.3).

The production of health-promoting FFs enriched with protein hydrolysates is highly strain-specific, as not all LAB possess the enzymatic machinery necessary for effective protein degradation. This diversity comes from variations in genetic makeup, particularly in the composition and activity of CEPs, peptide transport systems, and intracellular peptidases, which collectively influence peptide-release profiles. LAB mostly reported for their effective peptide release are *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *delbrueckii*, and *Lactococcus lactis* (Hernández-Ledesma et al., 2011; Raveschot et al., 2020). Strain selection is therefore essential to harness the full potential of LAB in developing value-added, protein-based FFs (Hayes & García-Vaquero, 2016).

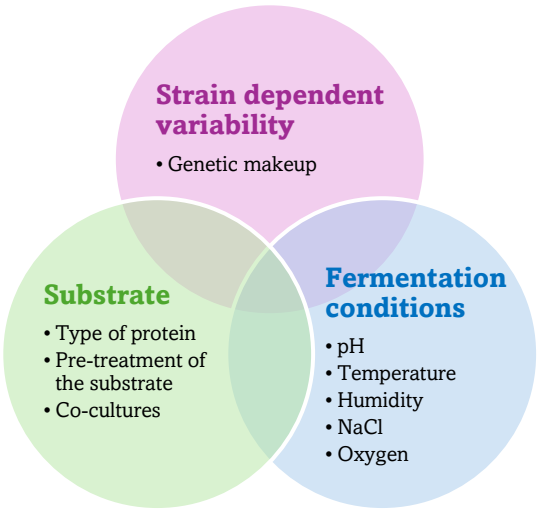

Figure 1.3 Parameters affecting microbial protein hydrolysis

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In addition to the intrinsic potential of each strain, the fermentation parameters, including temperature, pH, incubation time, relative humidity, NaCl, and oxygen availability, play a crucial role in shaping the proteolytic outcome. These conditions affect microbial growth and metabolism, the expression of proteolytic genes, and the rate of peptide release (Cruz-Casas et al., 2021). For example, extended fermentation time or specific pH levels may promote bacterial autolysis, contributing to the release of additional bioactive compounds into the medium (Otte et al., 2011). Moreover, the interaction between strain and protein substrate significantly influences the quality and amount of the resulting peptides. Certain LAB strains exhibit substrate preferences or synergistic behaviors when co-cultured with other microorganisms. Also, substrate pre-treatment can influence bacterial metabolism (Canon et al., 2020; Pessione & Cirrincione, 2016; Schär-Zammaretti et al., 2005). Given this complexity, selecting the best strain-substrate combination and the strategic optimization of process parameters are crucial for developing FFs enriched with protein hydrolysates with targeted bioactivities. Advanced tools such as proteomics, peptide mapping, and bioinformatics enable detailed characterization of peptide profiles, supporting the precise formulation of fermented protein hydrolysates aimed at specific health outcomes (Korhonen & Pihlanto, 2006).

1.2.4 LAB-driven Fermentation to Boost Functional Properties of Proteins

The *in situ* hydrolysis of proteins during fermentation allows the development of protein-based FFs enriched in bioactive compounds, potentially enhancing protein nutritional quality and functional properties. Table 1.1 presents a summary of recent studies conducted over the past five years that investigate the effects of LAB on the hydrolysis of various animal- and plant-based proteins and their resulting health-promoting properties.

Traditionally, animal-derived proteins have received the most attention due to their high protein content, balanced composition of essential amino acids, and functional properties. Among these, dairy proteins are the most extensively studied and utilized substrates in LAB fermentation, due to their compatibility with LAB strains. For instance, commercial products such as *Calpis* and *Evolus* - fermented milk beverages produced in Japan and Finland, respectively - exemplify how LAB fermentation can yield *in situ* bioactive peptides that contribute to cardiovascular health through mechanisms such as the inhibition of angiotensin converting enzyme (ACE) (Linares et al., 2017). Studies showed that *Streptococcus thermophilus* fermentation of cheese whey can generate bioactive compounds with antioxidant and ACE-inhibitory activities *in vitro* (Solieri et al., 2022). Likewise, fermentation of milk proteins by *Lactobacillus delbrueckii*, *Lacticaseibacillus paracasei*, and *Lactobacillus helveticus* has demonstrated antidiabetic and antioxidant effects *in vitro* (Shirkhan et al., 2023). *In vivo* research further supports these findings: *Enterococcus faecium* and *Lacticaseibacillus rhamnosus* fermentation of milk has been linked to hypotensive effects (Jiang et al., 2024b), while *Lb. rhamnosus* alone has been shown to improve protein digestibility (Kim et al., 2023). Additionally, cheese whey fermented by *Lb. plantarum* and *Lb. delbrueckii* subsp. *bulgaricus* has been associated with antiallergic effects *in vivo* (Jiang et al., 2024a). Beyond dairy, other animal-derived proteins have also been explored. For example, *St. thermophilus* fermentation of fish proteins has produced antimicrobial and antioxidant activities *in vitro* (Phupaboon et al., 2023).

In recent years, growing interest in sustainability and alternative protein sources has driven research toward the application of LAB fermentation to less conventional substrates, such as edible insects. Insect proteins are particularly promising due to their high nutritional value, environmental sustainability, and emerging evidence supporting their capacity to generate biologically active peptides through fermentation (Ma et al., 2023). *Lc. lactis* showed potential to enrich a mealworm flour with antioxidant and ACE-inhibitory peptides (Mendoza-Salazar et al., 2021). Nevertheless, consumer acceptance remains a major barrier in this context, as insect-based products are often perceived as unfamiliar or unappealing in many cultures.

Table 1.1 *In vitro*/*in vivo* studies from the last 5 years examining the effect of LAB protein hydrolysis of animal and plant-based proteins on their health-promoting properties

Strain	Protein source	*In vitro*/ *in vivo*	Health-promoting effect	Reference
St. thermophilus	Cheese whey	*In vitro*	Antioxidant and ACE-inhibitory activities	(Solieri et al., 2022)
Lb. delbrueckii *Lb. paracasei* *Lb. helveticus*	Milk	*In vitro*	Antidiabetic and antioxidant activities	(Shirkhan et al., 2023)
St. thermophilus	Fish	*In vitro*	Antimicrobial and antioxidant activities	(Phupaboon et al., 2023)
Lc. lactis	Worm flour	*In vitro*	Antioxidant and ACE-inhibitory activities	(Mendoza-Salazar et al., 2021)
Lb. delbrueckii	Soy	*In vitro*	Antioxidant and ACE-inhibitory activities	(Chourasia et al., 2022a)
Lb. plantarum *Lb. acidophilus*	Almond	*In vitro*	Antioxidant, ACE-inhibitory, and anti-inflammatory activities	(Areche et al., 2025)
Lb. plantarum *Lb. delbrueckii* subsp. *bulgaricus*	Cheese whey	*In vivo*	Antiallergic effect	(Jiang et al., 2024a)
Ent. faecium *Lb. rhamnosus*	Milk	*In vivo*	Hypotensive effect	(Jiang et al., 2024b)
Lb. rhamnosus	Milk	*In vivo*	Increased protein digestibility	(Kim et al., 2023)
Lb. brevis	Pea	*In vivo*	Sleep-enhancing activity	(Kim et al., 2025)
Lb. paracasei *Lb. plantarum*	Wheat protein	*In vivo*	Antifatigue effect and gut microbiota modulation	(Zhang et al., 2022)
Lb. plantarum	Soy	*In vivo*	Anti-aging effect	(Zhou et al., 2021)

At the same time, plant-based proteins have become central in the development of FFs. The world demand for plant-based alternatives is noticeably increasing due to the rise of ethical, health, and environmental concerns among consumers. While plant proteins typically exhibit lower digestibility and less favorable amino acid profiles compared to animal proteins, LAB fermentation has proven to be a powerful tool to improve plant protein digestion and adsorption (Wu et al., 2025). Also, numerous studies have explored fermentation as a method to enhance the flavor of plant proteins (Shi et al., 2021), yet research on the LAB role in improving their physiological properties through fermentation remains limited. *In vitro* studies demonstrated that *Lb. delbrueckii* fermentation of soy protein resulted in antioxidant and ACE-inhibitory activities (Chourasia et al., 2022a), while *Lb. plantarum* and *Lb. acidophilus* fermentation of almond proteins generated antioxidant,

ACE-inhibitory, and anti-inflammatory effects (Areche et al., 2025). *In vivo* studies also highlight beneficial physiological effects from fermented plant proteins. For example, *Levilactobacillus brevis* fermented pea protein was associated with sleep-enhancing activity (Kim et al., 2025). *Lb. paracasei* and *Lb. plantarum* fermentation of wheat protein demonstrated antifatigue effects and modulation of gut microbiota (Zhang et al., 2022), while *Lb. plantarum* fermented soy protein exhibited anti-aging properties (Zhou et al., 2021). Overall, both traditional animal and plant protein sources have been successfully used as substrates for FFs enriched with protein hydrolysates. Antioxidant and ACE-inhibitory activities remain the most reported health-promoting properties *in vitro*. At the same time, *in vivo* studies also reveal broader physiological benefits, including hypotensive, antiallergic, anti-aging, sleep-enhancing, and gut microbiota-modulating effects.

Protein hydrolysates are increasingly recognized for their potential to modulate gut microbiota composition. While most research on microbiota modulation has centered on dietary polysaccharides and polyphenols, emerging evidence suggests that some peptides can resist digestion in the upper gastrointestinal tract and reach the colon intact, where they may exert selective, prebiotic-like effects on microbial populations (Wang et al., 2022). For example, low-molecular-weight peptides (<1 kDa) derived from whey protein hydrolysates have been shown to significantly enhance the relative abundance of beneficial bacterial genera such as *Lactobacillus* spp., *Bifidobacterium* spp., and members of the *Bacteroidetes* phylum (Yu et al., 2016). Similarly, casein-derived hydrolysates have been reported to promote the growth of *Lactobacillus* spp., while simultaneously suppressing potentially pathogenic *Bacteroides* spp., suggesting a role in maintaining microbial balance in the gut (Visser et al., 2012). Notably, peptides are often preferentially utilized by gut microbes over free amino acids, particularly when carbohydrate sources are depleted. Microbial metabolism of these peptides leads to the production of key metabolites such as branched-chain amino acids, short-chain fatty acids (SCFAs), and branched short-chain fatty acids (BCFAs), which contribute to the maintenance of gut microbial homeostasis and overall gut health (Hu et al., 2024). In addition to the peptides themselves, LAB viable in fermented hydrolysates can also play a pivotal role in shaping the gut microbiota. These LAB strains may survive gastrointestinal transit and temporarily colonize the colon, where they compete with pathogens, enhance mucosal immunity, and influence microbial community structure through the production of antimicrobial compounds and metabolic cross-feeding, supporting a healthy microbial ecosystem (Cunningham et al., 2021).

1.3 *In vitro* and *in vivo* Approaches to Study Biological Functionalities of Protein-based FFs

Due to the multifactorial nature of FFs, which contain a heterogeneous mixture of bioactive compounds and live microorganisms, understanding their health effects necessitates a multi-level investigation combining *in vitro*, *in vivo*, and increasingly, *omic*-based methodologies.

In vitro methods are efficient, cost-effective, and reproducible approaches widely used as preliminary tools to investigate the biological functionalities of FFs. These models provide valuable insights into specific bioactivities under controlled conditions. Chemical assays, such as 2,2-Diphenyl-1-picrylhydrazyl (DPPH) or Ferric Reducing Antioxidant Power (FRAP) for antioxidant activity, and enzyme inhibition tests, including ACE-inhibition for antihypertensive potential, enable rapid screening of functional properties. Importantly, *in vitro* systems are designed to isolate and evaluate a single mechanism of action at a time, allowing researchers to attribute specific bioactivities within complex matrices. This mechanistic focus is beneficial for optimizing fermentation processes, as it supports the selection of microbial strains and fermentation parameters that most effectively enhance the desired functional properties of the final product (Benkhaira et al., 2021; Polli, 2008). The presence of LAB within FFs, along with functional metabolites, further enhances their functional profile. Their ability to survive gastrointestinal transit can be studied through *in vitro* gastrointestinal digestion models (Brodkorb et al., 2019), and the impact of the fermentates on gut microbiota and its metabolites can be assessed through *in vitro* fecal fermentation models (Pérez-Burillo et al., 2021).

Despite their utility, *in vitro* studies often lack the physiological complexity of living organisms and do not fully replicate the interactions between food components, host tissues, and the gut microbiota. As a result, bioactivities observed *in vitro* may not always translate into meaningful effects *in vivo*. Therefore, findings from *in vitro* assays must be interpreted with caution and complemented by *in vivo* studies to confirm biological relevance and assess bioavailability, metabolism, and systemic effects under realistic conditions. *In vivo* studies, primarily animal models or human trials, include evaluating the impact of FFs enriched with protein hydrolysates on parameters such as blood pressure, glucose metabolism, lipid profiles, systemic inflammation, and gut microbiota composition (Xu et al., 2021). Nonetheless, *in vivo* studies are expensive, time-consuming, and ethically demanding, with limited suitability for high-throughput screening across multiple conditions or formulations. Consequently, starting the evaluation of the health-promoting properties of FFs with *in vitro* models remains a necessary and strategic step (Polli, 2008). This approach allows for the efficient identification of promising strains, fermentation parameters, and bioactive profiles, which can then be prioritized for *in vivo* validation.

Lastly, *omic* technologies - including metagenomics, transcriptomics, proteomics, and metabolomics - have become indispensable tools for unraveling the complex interactions between FFs, the host, and the gut microbiota. Significantly, *omic* tools can be applied to both *in vitro* and *in vivo* settings. Metagenomic or whole-genome sequencing, for example, can be employed to investigate how FFs modulate the composition of the gut microbiota. In parallel, bioinformatics allows for the identification of correlations between specific microbial taxa and metabolites derived from fermentation or digestion (Pérez-Burillo et al., 2021; Sánchez et al., 2013). Transcriptomic and

proteomic analyses are particularly valuable for exploring the metabolic activity of the microbial strains during fermentation, helping to optimize processing conditions and maximize the production of beneficial compounds (Li et al., 2021b). Furthermore, these techniques can be applied to study how microbial metabolism and gene expression change during gastrointestinal digestion, offering insights into the stability, transformation, and bioavailability of probiotics and related functional molecules (Stastna, 2024). These *omic*-based approaches provide a robust framework for the development of FFs with targeted health benefits.

1.4 Fermentation of Alternative Protein Substrates: Innovative Aspects and Challenges

The revival of fermentation using autochthonous bacterial strains represents a frontier in the functional valorization of protein-based matrices. These native strains, shaped by years of spontaneous fermentation in specific environments, often exhibit superior health-promoting features compared to standardized industrial cultures. However, the application of these strains carries significant challenges: isolating the most promising candidates, characterizing their metabolic capacities, and defining optimal fermentation parameters require precise and strain-specific approaches (Grujović et al., 2019). The complexity of microbial metabolism and its modulation with diverse protein substrates further complicates this task.

This opportunity intersects with one of the most vital issues in modern food systems: the growing demand for sustainable, high-quality protein. Proteins are fundamental to human nutrition, and animal-based sources remain highly valuable due to their complete amino acid profile and high bioavailability. However, their production is associated with relatively higher environmental impacts, including greenhouse gas emissions, land use, and water consumption. In this context, plant-based proteins are gaining prominence as complementary and more sustainable alternatives. The gradual shift toward a balanced combination of animal- and plant-based proteins is commonly referred to as the *protein transition* (Duluins & Baret, 2024). In parallel, the valorization of animal-derived by-products, such as whey from cheese production or residual tissues from meat processing, represents an underexploited opportunity to recover high-value proteins. When subjected to microbial fermentation, these substrates can be transformed into functional foods or ingredients with improved health-promoting features (Kuo et al., 2024; Sabater et al., 2020). This circular approach not only reduces food waste but also contributes to the development of novel health-oriented foods. Furthermore, the development of hybrid protein products - combining plant and animal proteins - represents a strategy to support the protein transition (Olivas et al., 2024). Such formulations can enhance the nutritional quality of protein food products while reducing the environmental footprint, and fermentation plays a central role in optimizing their functional and sensorial characteristics.

Overall, the strategic application of fermentation with carefully selected native microbial strains offers a robust and sustainable approach for improving the biological value of diverse protein sources. This approach directly supports several goals of the 2030 Agenda for Sustainable Development (a global action plan to promote peace and prosperity for people and the planet adopted by all United Nations Member States in September 2015 and to be implemented by 2030), particularly Goal 2 (Zero Hunger), Goal 3 (Good Health and Well-being), and Goal 12 (Responsible Consumption and Production), by promoting health-oriented and environmentally responsible food systems.

1.5 Aim and Outline of the PhD Thesis

The overall aim of this PhD project was to explore the potential of autochthonous LAB to generate health-promoting properties from food proteins through fermentation. The work was designed to investigate how LAB can act as biotechnological tools to transform different protein sources into functional and sustainable FFs. To address this aim, whey proteins were used as a model of animal-derived proteins, while pea proteins served as a model for plant-based substrates, reflecting both the importance of valorizing agri-food by-products and the growing interest in diversifying protein sources toward more sustainable food systems. The thesis is structured into six experimental chapters, each addressing specific research goals that collectively contribute to the general objective.

Chapter 2 focused on the identification and screening of autochthonous LAB isolated from naturally fermented dairy and plant-based products. LAB strains originating from dairy matrices were screened in whey-based systems, while those isolated from plant matrices were evaluated in pea-based systems. This approach enabled the selection of strains with proteolytic potential on food proteins and resulted in a database of LAB strains capable of producing protein fermentates enriched in low-molecular-weight peptides, laying the foundation for subsequent investigations.

Chapter 3 was dedicated to the biochemical characterization and functional evaluation of fermentates obtained from whey proteins. The primary objective was to evaluate the capacity of LAB fermentation to enhance the health-promoting properties of whey-based substrates, focusing on antioxidant, ACE-inhibitory, and antimicrobial activities, as well as its impact on gut microbiota composition and the resulting metabolites. In parallel, ***Chapter 4*** investigated the fermentates resulting from pea proteins, using the same experimental framework to explore the role of LAB fermentation in enhancing the potential health-promoting properties of pea-based protein matrices.

The results from the first chapters led to the identification of a promising strain, *Lacticaseibacillus paracasei* AF43, originally isolated from a dairy matrix and selected for its functional potential after whey protein fermentation. The strain was therefore chosen for in-depth analyses, which were conducted in collaboration with the Norwegian University of Life Sciences. Building on this,

Chapter 5 aimed to elucidate the metabolic behavior of *Lb. paracasei* AF43 when grown in different protein-based environments: whey, pea, and a combination of both. Proteomic and biochemical analyses were integrated to link metabolic responses with functional outcomes, providing mechanistic insights into how diverse protein substrates shape the strain's ability to develop potential health-promoting FFs.

Chapter 6 further investigated the adaptive response of *Lb. paracasei* AF43 during simulated gastrointestinal digestion. The goal was to evaluate the strain's ability to survive and maintain metabolic functionality after exposure to digestive conditions, providing insights into its potential contribution to gut health and its suitability for application in functional FFs.

Finally, ***Chapter 7*** integrates and discusses the overall findings of the thesis, highlighting the innovative aspects of the work and outlining future research perspectives.

Chapter 2:
Building the LAB Strain Database

2.1 Introduction

Among fermented foods (FFs), those derived from natural or spontaneous fermentation stand out for their rich microbial diversity and unique biochemical profile. Unlike defined starter cultures, which offer predictability and reproducibility, spontaneous fermentation relies on complex, autochthonous microbial populations originating from raw materials and processing environments. These undefined communities yield products with distinctive regional character and flavor, often protected by traditional practices or geographical designations (Rossi et al., 2024). A central player in these ecosystems is lactic acid bacteria (LAB), which dominates lactic acid fermentation. Several factors, such as substrate composition, temperature, and process conditions, shape microbial biodiversity in artisanal FFs.

In dairy products, LAB diversity is pronounced and dynamic, influenced not only by the nature of the starter culture but also by additional factors such as milk origin, animal feeding, time of fermentation, and, in the case of cheese, ripening parameters (Posheva et al., 2024). In this context, LAB are categorized into two groups: starter LAB (SLAB) and non-starter LAB (NSLAB). SLAB are composed of strains of *Lactococcus lactis*, *Streptococcus thermophilus*, and other acid-producing species. Their primary role is to rapidly ferment lactose into lactic acid, lowering pH, and contributing to the initial flavor development. NSLAB include species such as *Lacticaseibacillus paracasei*, *Lactiplantibacillus plantarum*, and *Lacticaseibacillus rhamnosus*. Though initially present in low numbers, NSLAB become dominant during ripening due to their ability to survive harsh conditions (e.g., low pH, high salt) and contribute to the development of complex flavors, aroma, and texture through proteolysis and lipolysis (Beresford et al., 2001; Grujović et al., 2022).

Among plant-based FFs, vegetable fermentations (e.g., sauerkraut, fermented cabbage) are characterized by LAB communities shaped by a variety of factors, including plant species, environmental conditions, and harvesting practices. Although LAB represent only a minor component (2 - 4 log cfu/g) of the native microbiota, they play a central role in spontaneous vegetable fermentation when conditions such as oxygen, temperature, and salt concentration are favorable (Di Cagno et al., 2013). *Lb. plantarum*, *Leuconostoc*, *Pediococcus,* and *Weissella* species prevail under varying conditions (Şanlier et al., 2019). As for sourdough, it is defined as the spontaneous fermentation of a mixture of flour and water and supports diverse LAB species, which are strongly influenced by fermentation conditions such as time, temperature (from 22 to 40 °C), and substrate (e.g., cereals or pulse flour) (Arora et al., 2021).

In these products, LAB are essential not only for acidification, safety, and flavor development, but also for their emerging health-promoting properties. Many LAB strains from natural ecosystems exhibit vitamin biosynthesis (mainly B-group), production of bioactive compounds (e.g., γ-

aminobutyric acid, exopolysaccharides, conjugated linoleic acid, angiotensin-converting enzyme (ACE)-inhibitory peptides), and potential probiotic activity (Şanlier et al., 2019).

Recognizing the biological potential of naturally occurring LAB, this PhD project focused on their application in the valorization of underutilized substrates. In particular, attention was directed toward protein-based by-products like cheese whey and emerging plant-based protein sources such as pea (*Pisum sativum* L.). Whey, traditionally considered a by-product, is a highly valuable resource rich in protein (0.6–1 %) and other nutrients like lactose, making it an attractive substrate for fermentation. While commonly processed through physicochemical methods (e.g., filtration) for protein concentrates or industrial lactose conversion, biological treatments like microbial fermentation to enhance whey for food applications remain underexplored (Khurana et al., 2023). At the same time, demand for plant-based proteins is rapidly growing in response to the ongoing protein transition (Duluins & Baret, 2024). Pea is gaining prominence due to its gluten-free and low allergenicity features. Furthermore, it is cost-effective and high in protein (20-30 %) and carbohydrates (60-65 %), and low in fat (1.5-2 %) (Shi et al., 2021). In the market, pea protein comes in different forms, including flour, concentrate, and isolate, depending on the level of protein purification. Both conventional and innovative processing methods, including fermentation, have been studied to improve the flavor and technological functionality of pea proteins (Shi et al., 2021), yet research on LAB's role in enhancing their biological properties remains limited. In these protein-based substrates, one of the main metabolic pathways activated by LAB is proteolysis. This process leads to the hydrolysis of proteins, resulting in the release of peptides and free amino acids. Some of these peptides, generated *in situ*, have been associated with bioactive functions, such as antihypertensive, antioxidant, immunomodulatory, and antimicrobial effects, thereby enhancing the biological potential of the FF matrix itself. These transformations are mediated by the proteolytic system of LAB, although not all LAB strains possess the complete enzymatic machinery required for effective protein degradation and peptide release (Ter et al., 2024).

Given these considerations, the starting point of this PhD thesis was to isolate and identify LAB from naturally fermented dairy and plant-based products. Then, LAB isolates were screened for the ability to grow on and hydrolyze whey and pea proteins, also evaluating their capacity to release peptides into the fermentation medium. Subsequently, DNA fingerprinting and typing were performed to build a database of peptide-releasing LAB strains with distinct molecular patterns.

2.2 Materials and Methods

2.2.1 Materials

Maximum Recovery Diluent (MRD), De Man, Rogosa, and Sharpe (MRS) broth, M17 broth, Skim Milk Powder, and Bacteriological agar were purchased from Oxoid (Milan, Italy). Delvocid® was

obtained from DSM Food Specialties (Delft, the Netherlands), and a Gram Color Kit from Liofilchem Diagnostics (Teramo, Italy). Hydrogen peroxide, glycerol, agarose, Tris/Borate/EDTA buffer (TBE), yeast extract, lactose, casein hydrolysate, glucose, sodium tetraborate decahydrate, sodium dodecyl sulfate, dithiothreitol, o-phthaldialdehyde (OPA), absolute ethanol, trichloroacetic acid, leucine, hydrochloric acid, sodium hydroxide, acetonitrile, trifluoroacetic acid (TFA), bovine serum albumin (BSA), β-lactoglobulin (β-Lg), α-lactalbumin (α-La), caseinomacropeptide (CMP), aprotinin from bovine lung, C-peptide, and L-glutathione oxidized were purchased from Merck (Darmstadt, Germany). The InstaGene™ extraction kit was obtained from Biorad (Hercules, CA, USA), and PCR amplification reagents, primers, and GeneRuler DNA ladders 100 bp and 1 kb from Thermo Fisher Scientific (Waltham, MA, USA). QIAquick PCR Purification Kit was purchased from Qiagen (Hilden, Germany). Whey protein concentrate (WPC; 77 % protein) was sourced from Bulk™ (Colchester, UK), and pea protein concentrate (PPC; 80 % protein) from Raab Vitalfood (Rohrbach, Germany).

2.2.2 Isolation and Phenotypic Characterization of Bacterial Strains

Bacterial strains were isolated from raw milk cheeses (n=21), natural milk cultures (n=8), natural cheese whey (n=1), fermented *Brassica rapa* (n=2), sauerkraut (n=2), sourdough (n=1), and water kefir (n=1) collected from artisanal productions in Northeastern Italy. For the isolation of the cultures, 10 g of each sample was added with 90 mL of MRD in a stomacher bag and homogenized for 2 min in a BL Smart Lab (Astori Tecnica, Brescia, Italy). Then, the suspension was serially diluted and spread-plated onto MRS agar, incubated at 30 °C and 37 °C for 48 h under anaerobic conditions, and M17 agar (supplemented with 10 % v/v lactose), incubated at 37 °C for 48 h. As for plant-based products, isolation was carried out from count plates of MRS agar added with 25 mg/L of Delvocid® and incubated at 30 °C for 48 h under anaerobic conditions. Then, three to eight morphologically unique colonies from each sample were randomly picked up and purified onto MRS or M17 agar plates. The isolated colonies were further analyzed for Gram staining and catalase activity (3 % hydrogen peroxide). The Gram-positive and catalase-negative isolates were propagated in MRS or M17 broth and stored at -80 °C with glycerol (30 % v/v) as a cryoprotectant.

2.2.3 Molecular Identification of LAB Strains

Two hundred three isolates were grown in MRS or M17 broth; then, the DNA extraction was performed with the InstaGene™ extraction kit following the manufacturer's instructions. DNA concentration and purity were measured by NanoDrop® (Thermo Fisher Scientific, Waltham, MA, USA) and checked for integrity by agarose gel electrophoresis, using a 0.7 % agarose gel in TBE buffer 0.5x. Then, DNA was standardized to a concentration of 100 ng/μL. Amplification of the 16S rRNA gene was carried out by PCR using primers P1V1 (5'-GCGGCGTGCCTAATACATGC-

3') and P4V3 (5′-ATCTCAGCATTTCACCGCTAC-3′) (Klijn et al., 1991). Each reaction mixture (50 µL) contained 1x reaction buffer, 3 mM $MgCl_2$, 0.2 mM of dNTPs, 0.2 µM of each primer, 0.025 U of Taq DNA Polymerase, and 1 µL of bacterial DNA. The PCR cycle consisted of an initial denaturation step (5 min at 95 °C), followed by amplification for 35 cycles as follows: denaturation at 95 °C for 1 min, annealing at 42 °C for 1 min, extension at 72 °C for 1.5 min, and a final extension step at 72 °C for 7 min in a SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific). The amplicons were separated in a 2 % agarose gel in TBE buffer 0.5x with 100 bp DNA ladder as a size marker. Amplicon purification was performed with a QIAquick PCR Purification Kit following the manufacturer's instructions. The DNA was standardized to a concentration of 5 ng/µL. The 16S rRNA sequencing was performed by Eurofins Genomics (Ebergsberg, Germany). To determine the closest known relatives of the isolates, the sequences were aligned with the contents of GenBank using the Blast algorithm (https://blast.ncbi.nlm.nih.gov/).

2.2.4 Fingerprint Typing of the Selected LAB Strains

The Random Amplified Polymorphic DNA (RAPD) fingerprinting was used to identify unique strains among LAB isolates. Two primers were used to set up the reactions: D11344 (5'-AGTGAATTCGCGGTCAGATGCCA-3') and D8635 (5'-GAGCGGCCAAAGGGAGCAGAC-3'). For both primers, the following amplification conditions were used: an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 40 °C for 1 min, and extension at 72 °C for 1.5 min. The final extension was performed at 72 °C for 10 min. The reaction mixture (25 µL) consisted of reaction buffer 1x, 0.25 mM of dNTPs, 0.8 µM of the primer, 3 mM of $MgCl_2$, 1 U of *Taq* DNA Polymerase, and 1 µL of bacterial DNA (Andrighetto et al., 2002). PCR profiles were visualized after 3 h of gel electrophoresis at 80 V in a 1.5 % agarose gel in TBE buffer 0.5x. The 1 kb DNA ladder was used as a size marker. Grouping of the RAPD-PCR profiles was performed using the Gel Compare 6.1 software package (Applied Maths, Sint-Martens-Latem, Belgium) by applying the Pearson correlation similarity coefficient and the UPGMA cluster analysis (Renoldi et al., 2024).

2.2.5 Screening of Dairy-derived LAB Isolates

2.2.5.1 Qualitative Screening of Proteolytic Activity on Skim Milk Agar

A preliminary screening for proteolytic activity of the isolates of dairy origin was carried out on Skim milk agar (Skim Milk Powder 28 g/L, casein hydrolysate 5 g/L, yeast extract 2.5 g/L, glucose 1 g/L, agar 15 g/L, pH 7.0 ± 0.2) (Renoldi et al., 2024). Cultures were reactivated in 1 mL of MRS or M17 broth and incubated at 30 °C or 37 °C for 24 h. 5 µL of each culture was then deposited onto Skim milk agar plates and incubated at 30 °C or 37 °C for 72 h under anaerobic conditions. The appearance of a clear halo around the spot indicated the presence of proteolytic activity. The

test was performed in duplicate. As a positive control, the strain *Pseudomonas fluorescens* DIAL22 was used (Innocente et al., 2019).

2.2.5.2 Assessment of Growth Kinetics and Proteolytic Activity in Whey Medium

The dairy isolates were tested to assess growth capacity and proteolytic activity in whey medium (WM). To prepare WM, WPC (15 g/L) and lactose (50 g/L) were dissolved in water (pH 6.5 ± 0.1) (Solieri et al., 2022). The suspension was stirred for 1 h at 4 °C, then centrifuged at 5,000 × *g* for 10 min at 4 °C. The supernatants were subsequently filtered twice through 0.45 µm and 0.22 µm polystyrene filters (Minisart™, Sartorius, Göttingen, Germany) and analyzed as follows.

i. Turbidimetric growth curves were used to evaluate the ability of the LAB strains to grow in WM. The overnight culture of each strain was centrifuged (13,000 × *g* for 5 min), washed twice, and resuspended in 1 mL of MRD. The strains were inoculated into 96-well U-bottomed microplates, with each well containing 10 µL of a 1:100 diluted culture (final concentration approximately 5 x 10^4 cfu/mL) and 190 µL of WM. Control wells containing inoculated MRS or M17 broth were prepared for each strain. Blank wells containing uninoculated media were also prepared. Microbial growth was monitored for 72 h at 30 °C or 37 °C. The optical density at 630 nm was recorded every 30 min, after shaking for 5 s, using a Sunrise microplate reader (Tecan, Milan, Italy). All tests were performed in duplicate.

ii. The degree of hydrolysis of whey proteins after fermentation was determined with the OPA method (Church et al., 1983). 10 µL of the overnight culture of each strain was inoculated into 1 mL of WM and incubated at 30 °C or 37 °C for 72 h. The cell-free supernatant, obtained after centrifugation at 5,000 × g for 5 min, was used for the analysis. 100 mL of the OPA solution was prepared, containing 3.813 g of sodium tetraborate decahydrate, 0.1 g of sodium dodecyl sulfate, 0.088 g of dithiothreitol, and 0.08 g of OPA, previously dissolved in 2 mL of absolute ethanol. The OPA solution was kept in the dark and used within 2 h. 0.75 M trichloroacetic acid was added to each cell-free supernatant in a volumetric ratio of 1:3, and after centrifugation (for 10 min at 5,000 × *g*), the supernatant was collected. 3 mL of OPA solution and 150 µL of the sample were placed in a quartz cuvette. After incubation in the dark for 2 min at room temperature, a spectrophotometric reading was taken at 340 nm in a UV spectrometer 2501PC (Shimadzu, Kyoto, Japan). The data were converted to mM leucine using a calibration curve obtained with aqueous solutions of leucine (0.05-2.5 mM). The degree of hydrolysis was estimated against a sample called TQ, corresponding to 100 % hydrolysis of WM in 6 M hydrochloric acid at 110 °C for 24 h. After neutralization with 7.5 mL of 2 M sodium hydroxide, the solution was centrifuged at 10,000 × *g* for 30 min, and the supernatant was analyzed with the OPA method. The degree of hydrolysis (DH%) was calculated according to Eq. 1:

$$DH\% = \frac{C\_sample}{C\_TQ} x100 \qquad \text{(Eq. 1)}$$

Where C_sample is the concentration of leucine (mM) of the sample under examination, and C_TQ is the concentration of leucine (mM) of the TQ solution.

iii. The extent of protein hydrolysis and the molecular weight (MW) distribution of peptides released during fermentation were estimated using Size-Exclusion High Performance Liquid Chromatography (SE-HPLC), as described by Cui et al. (2022) and Innocente et al. (2023). A TSKgel 2000 SWXL 300mm x 7.8 mm column (Tosoh Bioscience, Griesheim, Germany) with a mobile phase consisting of water/acetonitrile/TFA (55/45/0.1, v/v/v) was used. The flow rate was 0.5 mL/min, and the column temperature was maintained at 30 °C. A 10-µL sample was injected into a LC-4000 HPLC system (Jasco Europe, Lecco, Italy) equipped with a PDA detector set at 220 nm. BSA (66,463 Da), β-Lg (18,400 Da), α-La (14,000 Da), CMP (6,800 Da), aprotinin (6,511 Da), C-peptide (3,183 Da), L-glutathione oxidized (612 Da), and L-Serine (106 Da) were run as standards. Data analysis was performed using the chromatography software ChromNAV2 (Jasco Europe). The extent of protein hydrolysis (PH%) was evaluated by measuring the reduction in whey protein area following fermentation. PH% was expressed using the formula shown in Eq. 2:

$$PH\% = \frac{(Area\_WM - Area\_F)}{Area\_WM} \, x \, 100 \qquad \text{(Eq. 2)}$$

Where Area_WM and Area_F represent the chromatographic peak areas of the WM and fermentates, respectively. These areas refer to the sum of all main protein peaks (BSA, α-La, β-Lg, and CMP).

Subsequently, MW distribution analysis was performed to estimate the content of peptides within the 6-3 kDa and <3 kDa ranges. To this purpose, a calibration curve was obtained by injecting MW standards at different concentrations, from 0.1 to 5 mg/mL.

2.2.6 Screening of Plant-derived LAB Isolates

2.2.6.1 Assessment of Growth Kinetics and Proteolytic Activity in Pea Medium

The plant-based LAB isolates were tested to assess growth capacity and proteolytic activity in pea medium (PM). To prepare PM, PPC (15 g/L) and glucose (50 g/L) were dissolved in water (pH 6.5 ± 0.1). The suspension was stirred for 1 h at 4 °C, then centrifuged at 5,000 × *g* for 10 min at 4 °C. The supernatants were subsequently filtered twice through 0.45 µm and 0.22 µm polystyrene filters and analyzed as follows.

Turbidimetric growth curves of plant-based LAB isolates in PM, as well as the degree of hydrolysis (DH%), the extent of protein hydrolysis (PH%), and the peptide release after fermentation, were evaluated as described in Section 2.2.5.2.

2.2.7 Statistical Analysis

All trials were carried out at least in duplicate, and values were expressed as the means ± standard deviation. Statistical analysis was performed using R v 4.3.0 (The R Foundation for Statistical Computing, Vienna, Austria). Bartlett's test was used to check the homogeneity of variance. The difference between means was calculated using a t-test and one-way analysis of variance (ANOVA), and the Tukey test was used to determine statistically significant differences among means (p<0.05).

2.3 Results and Discussion

2.3.1 Isolation and Identification of LAB Strains

In this study, a total of 203 Gram-positive and catalase-negative isolates were initially collected, with 175 confirmed as LAB based on 16S rRNA sequencing and exhibiting unique fingerprint profiles according to RAPD analysis (data not shown). Table 2.1 summarizes the distribution of LAB species isolated from various dairy and plant-based FFs.

Among dairy FFs, cow's milk cheese yielded the highest number of isolates (n=84), due to the relatively large sample size. *Lb. paracasei* was the most frequently identified species, followed by *Lb. rhamnosus*, *Lb. plantarum,* and *Pediococcus acidilactici*. NSLAB, particularly *Lb. paracasei*, predominated also in ripened sheep's milk cheese and washed rind cheese. This prevalence aligns with previous studies highlighting the relevance of NSLAB during cheese ripening, contributing to flavor and texture development (Renoldi et al., 2024). In contrast, SLAB such as *St. thermophilus* and *Lc. lactis* were less frequently detected. In ripened artisanal cheeses, the production process exposes microbes to harsh conditions, including heating, acidification, salt addition, and oxygen exposure, which SLAB cannot survive over time. As a result, SLAB populations decline rapidly, often within hours or days after cheese production. NSLAB, which are more resistant to environmental stresses and capable of growing under nutrient-limited conditions, gradually become the dominant microbial group in the mature cheese (Gatti et al., 2014).

Natural milk cultures, used as undefined starters in several Protected Designation of Origin cheeses, are obtained by incubating thermally treated milk at ~40 to 45 °C until the desired acidity is obtained. The microbial communities are shaped by the raw material and the thermal treatment, which selects LAB biotypes (Morandi et al., 2019). In this study, they presented a LAB profile dominated by thermophilic species such as *Lactobacillus delbrueckii* (subsp. *lactis*, *delbrueckii*, and

bulgaricus) and *St. thermophilus*, consistent with what was observed by Rossi et al. (2024). Additionally, *Lc. lactis* and *Lb. paracasei*, together with thermophilic *Lactobacillus* species, were isolated from natural cheese whey, which usually retains residual microorganisms from the cheese fermentation process (Carvalho et al., 2013b). These isolates represent a microbial community that includes both SLAB and NSLAB involved in cheese production (Carvalho et al., 2013b).

Table 2.1 LAB species isolated from various natural dairy and plant-based FFs

Origin	Species
Dairy FFs	
Cow's milk cheese	*Lb. paracasei* (n=47)
	Lb. rhamnosus (n=10)
	Lb plantarum (n=10)
	P. acidilactici (n=5)
	Lc. lactis (n=5)
	St. thermophilus (n=3)
	Ent. malodoratus (n=3)
	Lc. raffinolactis (n=1)
Sheep's milk cheese	*Lb. paracasei* (n=3)
	Lc. lactis (n=1)
	Lc. raffinolactis (n=1)
Washed rind cheese	*Lb. paracasei* (n=3)
	P. acidilactici (n=2)
	Lb. rhamnosus (n=2)
	Lb. plantarum (n=2)
Natural milk culture	*Lb. delbrueckii* subsp. *delbrueckii* (n=7)
	St. thermophilus (n=8)
	St. macedonicus (n=4)
	Lb. delbrueckii subsp. *lactis* (n=4)
	Lb. delbrueckii subsp. *bulgaricus* (n=4)
Natural cheese whey	*Lc. lactis* (n=3)
	Lb. paracasei (n=3)
	Lb. delbrueckii subsp. *bulgaricus* (n=2)
Plant-based FFs	
Sauerkraut	*Lb. brevis* (n=10)
	Lb. plantarum (n=4)
	Leuc. citreum (n=3)
	Lb. pentosus (n=2)
Fermented *Brassica rapa*	*Lb. plantarum* (n=8)
	P. pentosaceus (n=3)
	Lb. pentosus (n=2)
Water kefir	*Lb. paracasei* (n=1)
	P. acidilactici (n=1)
Sourdough	*P. pentosaceus* (n=3)
	Leuc. pseudomesenteroides (n=2)
	W. paramesenteroides (n=2)
	Leuc. citreum (n=1)

As for plant-based fermentations, sauerkraut was dominated by *Levilactobacillus brevis* (n=10) and *Lb. plantarum* (n=4), species well-known for their adaptability in vegetable fermentation (Di Cagno et al., 2013). Fermented *Brassica rapa* exhibited a similar profile with predominance of *Lb.*

plantarum (n=8) and *Pediococcus pentosaceus* (n=3), underlining the selective pressures of vegetable substrates that favor these acid-tolerant species.

Water kefir showed low diversity, with only *Lb. paracasei* and *P. acidilactici* isolated. These findings are consistent with previous research, which has also reported the predominance of *Lb. paracasei* in the microbial communities of water kefir (Arrieta-Echeverri et al., 2023).

Sourdough isolates included *P. pentosaceus*, *Weissella paramesenteroides*, *Leuconostoc citreum*, and *Leuconostoc pseudomesenteroides,* suggesting that fermentation had occurred at relatively low temperatures (around 22 °C). Previous studies have shown that at higher temperatures (e.g., 30 °C), *Lactiplantibacillus plantarum/pentosus* tend to dominate due to their acid tolerance and metabolic efficiency. In contrast, at lower temperatures (e.g., 22°C), the ecosystem becomes more diverse, with the presence of *Leuconostoc* spp., *P. pentosaceus*, and *Lb. rhamnosus*, among others (Munch-Andersen et al., 2024).

The observed variation in LAB species abundance across products is influenced by the number of samples analyzed: the higher count from cow's milk cheese contributed to a broader species representation compared to products with only one or two samples (e.g., water kefir). Overall, data highlight the adaptation of LAB communities based on the fermentation matrix.

This PhD project sought to valorize alternative and by-product protein sources via microbial fermentation, using whey protein as a model for animal-derived substrates and pea protein for plant-based ones. To assess growth and proteolytic potential in diverse protein substrates, dairy-derived strains were cultivated in whey protein, while plant-associated strains were tested in pea protein. This approach was designed to reflect the ecological origin of the strains, aligning each group with its native or typical substrate. By simulating physiologically familiar conditions, this strategy aimed at enhancing the probability of expressing proteolytic activity, as commonly observed in traditional FFs of either animal or plant origin.

2.3.2 Screening of Dairy-derived LAB Isolates

A total of 133 LAB isolates from dairy products were preliminarily screened for proteolytic activity using skim milk agar assay, a rapid plate-based method that allows for a qualitative assessment of proteolysis on milk proteins (Renoldi et al., 2024). The screening revealed that approximately 60 % of the strains exhibited proteolytic activity, as indicated by the formation of clear halos around the colonies (Figure 2.1).

All 17 isolates of *Lb. delbrueckii* exhibited proteolytic activity. This species is well known for its strong proteolytic capability and plays a crucial role in proto-cooperative interactions, such as those involved in yogurt fermentation, as well as in the generation of flavor compounds during the aging of dairy products (Buchin et al., 2017; Ge et al., 2024). Among the *St. thermophilus* isolates, 9 out of 11 demonstrated proteolytic activity, particularly those derived from natural milk cultures.

This species is typically homofermentative with strong acidifying properties, and prior studies have shown a close association between proteolytic and acidifying activities in this microorganism (Galia et al., 2009).

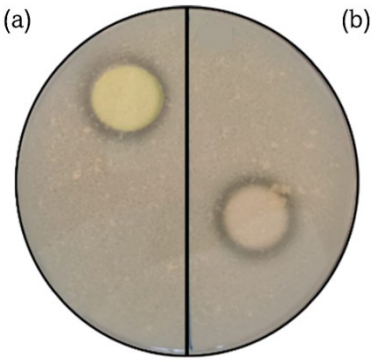

Figure 2.1 Proteolytic activity assay on Skim milk agar. (a) Positive control: *Ps. fluorescens* DIAL22; (b) *Lb. plantarum* L297

Approximately 70 % of the *Lb. paracasei* isolates, mostly originating from raw milk cheese, also displayed proteolytic activity. This observation is consistent with existing literature (Bottari et al., 2018; Galli et al., 2024; Renoldi et al., 2024), which describes *Lb. paracasei* as a species that adapts well to the dynamic ecosystem of cheese ripening, contributing to the development of the final sensory qualities of cheese through proteolysis.

Considering the results of the qualitative screening, the 80 proteolytic isolates were further tested for their ability to grow in WM, which contained whey protein as the primary nitrogen source. To evaluate the capacity to utilize whey proteins, turbidimetric growth curves were generated in WM, and MRS broth was used as a nutrient-rich control medium. Figure 2.2 illustrates representative growth curves of two contrasting strains.

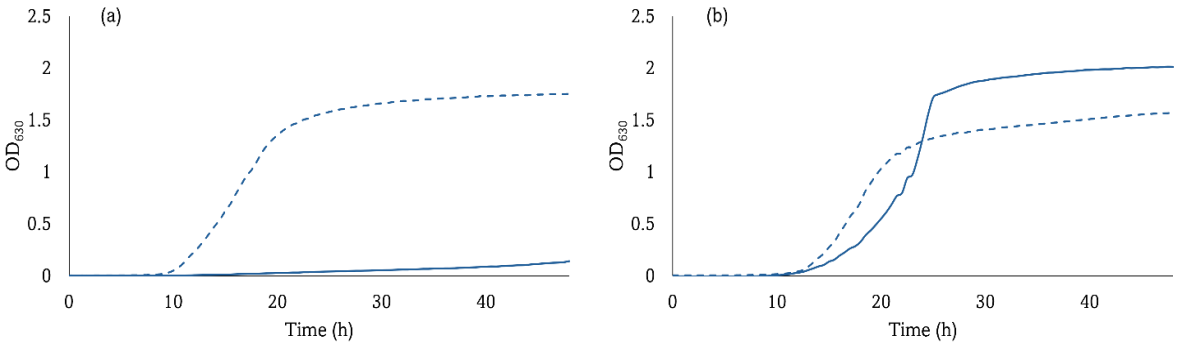

Figure 2.2 Growth curves of *Lb. paracasei* L325 (a) and *Lb. paracasei* L250 (b) in MRS (dotted line) and whey medium (WM) (continuous line)

In the case of *Lb. paracasei* L325, growth in WM was markedly slower than in MRS, with reduced absorbance values throughout the incubation period. This impaired growth could be attributed to the strain's limited ability to hydrolyze and assimilate whey proteins. Growth limitations in WM may be due not only to proteolytic inefficiency but also to metabolic constraints, such as the

inability of specific strains to utilize lactose, the primary carbohydrate in WM. Since lactose metabolism plays a central role in LAB energy production, especially in dairy environments, strains lacking β-galactosidase activity or appropriate transport systems may experience severely hindered growth in WM despite being proteolytically active (Kolev et al., 2022). Additionally, the potential inhibitory effect of some whey protein fractions cannot be excluded. Previous studies have suggested that whey proteins may exert mild antimicrobial effects, which could negatively influence the growth of susceptible strains (Innocente et al., 2023; Pescuma et al., 2008). Conversely, *Lb. paracasei* L250 showed a different pattern. Although growth in WM was initially slower, after approximately 24 hours, the absorbance values in the stationary phase exceeded those recorded in MRS. This observation indicates that the strain was not only capable of adapting to WM but also utilized its components more effectively than in MRS, reflecting a higher growth efficiency. These findings suggest that effective growth in whey-based media depends on a combination of factors, including proteolytic activity, lactose utilization, and the ability to tolerate or metabolize specific whey components. Strains exhibiting these traits could be promising candidates for the development of whey valorization strategies through fermentation.

Growth kinetics analysis revealed that 50 strains exhibited enhanced growth in WM compared to MRS, suggesting an efficient adaptation to and utilization of WM as a growth substrate. Table 2.2 shows the distribution of the strains capable of growing in WM. The most abundant species was *Lb. paracasei* (n = 12), all of which were isolated from raw-milk cheese. This dominance highlights the prevalence and adaptability of *Lb. paracasei* in cheese and its potential for growth in whey-based environments. Other relatively abundant species included *Lb. delbrueckii* subsp. *delbrueckii* and *St. thermophilus*, both mainly from natural milk cultures. Moderately represented species were *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. rhamnosus*, while several others, such as *P. acidilactici*, *Lb. plantarum*, *Lc. lactis*, *St. macedonicus*, and *Lb. delbrueckii* subsp. *lactis* were less frequent. Single isolates of *Lc. raffinolactis* and *Ent. malodoratus* were also identified.

Table 2.2 Proteolytic LAB strains capable of growth in whey medium (WM)

Species	Strains
Lb. paracasei (n=12)	A3, AF5, AF13, AF43, L121, L233, L177, L184, L250, L263, L309, L369
Lb. delbrueckii subsp. *delbrueckii* (n=7)	LbL2, LbL12, LbL14, LbL29, LbL31, LbL38, LbL39
St. thermophilus (n=7)	A1, L17, L24, L76, L112, L117, L122
Lb. delbrueckii subsp. *bulgaricus* (n=4)	LbL9, LbL36, LbL41, LbL42
Lb. rhamnosus (n=4)	AF3, AF11, AF34, L154
Lb. plantarum (n=3)	L241, L297, AF35
P. acidilactici (n=3)	L137, L185, AF27
St. macedonicus (n=3)	LbL43, LbL45, LbL47
Lc. lactis (n=3)	A5, AF45, AS16
Lb. delbrueckii subsp. *lactis* (n=2)	LbL33, LbL37
Lc. raffinolactis (n=1)	A406
Ent. malodoratus (n=1)	A2

The 50 strains capable of growing in WM were further quantitatively evaluated for proteolytic activity after 72 h of fermentation in WM, using the OPA method to quantify free amino groups. The resulting values were converted to the DH%, representing the proportion of free amino groups in the fermentates relative to a fully hydrolyzed WM, which was considered as 100 % hydrolysis. The results are presented in Figure 2.3. Unfermented WM showed a DH% of 1.36 ± 0.49 %. Among the tested strains, 18 induced a statistically significant increase (p<0.05) in DH%, with values ranging from 2.77 ± 0.06 % to 18.88 ± 1.50 %. These data are consistent with the literature (Chaudhari et al., 2017). Higher DH% values have been reported in fermentations involving yeast species. For instance, Dineshbhai et al. (2022) obtained DH% of approximately 23-48 % by fermenting three types of WPC with *Limosilactobacillus fermentum* KGL4 and *Saccharomyces cerevisiae* WBS2A. In this study, the strains that exhibited the highest degrees of hydrolysis belonged to the species *P. acidilactici*, *Ent. malodoratus*, *Lb. delbrueckii* (subsp. *delbrueckii*, subsp. *lactis*, and subsp. *bulgaricus*), *St. macedonicus*, *Lb. paracasei*, and *Lb. plantarum*.

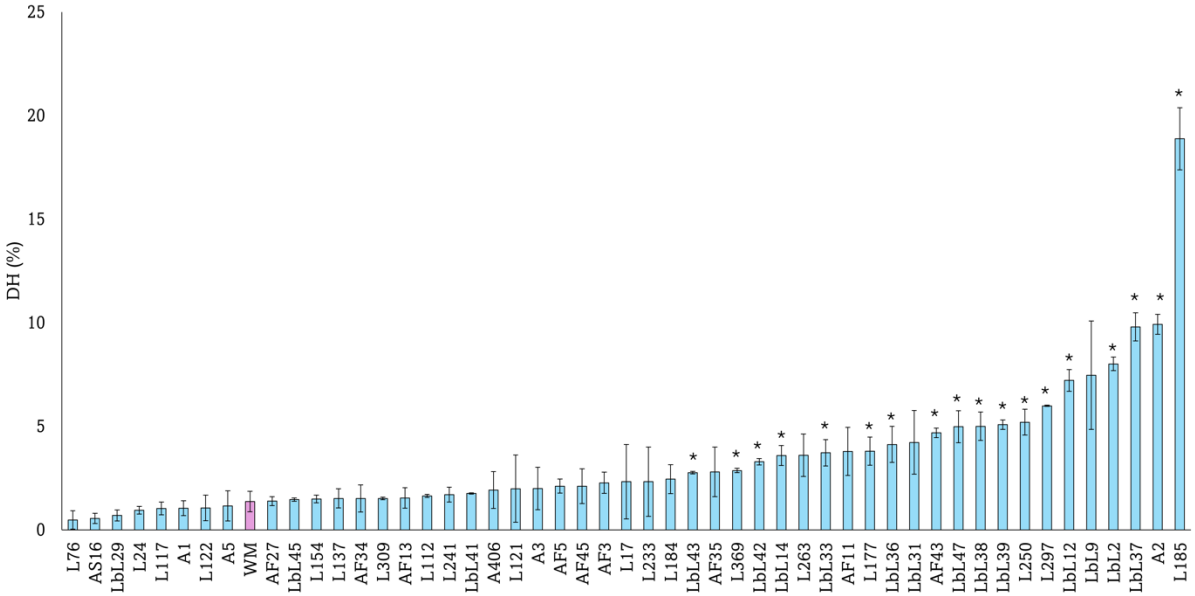

Figure 2.3 Results of the OPA analysis expressed as degree of hydrolysis (DH%). Data are expressed as mean ± standard deviation. Mean values marked with an asterisk are higher than the control (WM, shown in purple) (p<0.05)

As is widely known, LAB rely on proteolytic activity to obtain amino acids from external proteins. Cell-envelope proteinases (CEPs) generate peptides that may be transported into the cell and further degraded or remain in the medium if transport is inefficient. Additionally, low MW peptides may result from intracellular processing and be released during autolysis (Rizwan et al., 2023; Savijoki et al., 2006). Since peptides are generated during protein hydrolysis, excessive proteolysis may instead result in their degradation and loss (Solieri et al., 2022), Therefore, strains that exhibited a significantly higher DH% than the WM control (p<0.05) were further analyzed to assess peptide release by SE-HPLC, which separates macromolecules based on their MW.

Peptide fractions in the MW ranges of 6-3 kDa and <3 kDa isolates were quantified using a calibration curve. Importantly, bioactivities are generally reported to be highest for peptides with MW<0.8 kDa (i.e., <7 amino acids), and still significant for those with MW between 3-0.8 kDa. At the same time, those with extended chains are typically the least effective. For this reason, the fraction of peptides with MW<3 kDa is particularly noteworthy (Capriotti et al., 2015). Twelve of the 18 tested isolates released peptides in the <3 kDa range, indicating proteolysis and extracellular accumulation (Table 2.3). In contrast, peptides in the 6-3 kDa range decreased, suggesting that these were further degraded into smaller peptides or amino acids and consumed by the bacteria.

SE-HPLC analysis enabled the assessment of each microorganism's capacity to hydrolyze whey proteins. After fermentation, the PH% varied significantly among the tested strains, indicating that strains were able to hydrolyze whey protein differently (Table 2.3).

Table 2.3 Extent of protein hydrolysis (PH%) and concentration of peptides (mg/mL) in the 6-3 kDa and <3 kDa MW ranges released after 72-hour fermentation of WM. Values are presented as mean ± standard deviation

Strain	PH%	Peptide 6-3 kDa (mg/mL)	Peptide < 3 kDa (mg/mL)
WM	-	$1.65^{a} \pm 0.12$	$0.18^{d} \pm 0.04$
Lb. paracasei AF43	$21.84^{f} \pm 2.11$	$0.79^{b} \pm 0.11$	$0.39^{cd} \pm 0.01$
Lb. plantarum 297	$24.53^{f} \pm 2.29$	$1.04^{ab} \pm 0.11$	$0.47^{bd} \pm 0.04$
St.macedonicus LbL43	$25.72^{f} \pm 0.20$	$0.91^{b} \pm 0.04$	$0.49^{bd} \pm 0.06$
Lb. delbrueckii subsp. *delbrueckii* LbL38	$42.56^{bc} \pm 1.57$	$0.77^{b} \pm 0.02$	$0.56^{bc} \pm 0.08$
Lb. delbrueckii subsp. *bulgaricus* LbL9	$36.22^{d} \pm 3.39$	$0.81^{b} \pm 0.10$	$0.58^{bc} \pm 0.02$
Lb. delbrueckii subsp. *delbrueckii* LbL39	$46.13^{ab} \pm 1.31$	$0.78^{b} \pm 0.02$	$0.60^{bc} \pm 0.05$
Lb. delbrueckii subsp. *lactis* LbL37	$46.04^{ab} \pm 4.12$	$0.82^{b} \pm 0.00$	$0.64^{bc} \pm 0.01$
Lb. delbrueckii subsp. *lactis* LbL33	$47.67^{a} \pm 0.32$	$0.78^{b} \pm 0.01$	$0.62^{bc} \pm 0.06$
Ent. malodoratus A2	$31.25^{e} \pm 1.34$	$1.09^{ab} \pm 0.44$	$0.66^{bc} \pm 0.13$
Lb. delbrueckii subsp. *delbrueckii* LbL12	$40.85^{c} \pm 1.89$	$0.77^{b} \pm 0.02$	$0.71^{abc} \pm 0.05$
Lb. delbrueckii subsp. *delbrueckii* LbL14	$41.24^{c} \pm 0.44$	$0.74^{b} \pm 0.00$	$0.78^{ac} \pm 0.01$
Lb. delbrueckii subsp. *delbrueckii* LbL2	$32.02^{de} \pm 1.28$	$1.03^{ab} \pm 0.24$	$1.05^{a} \pm 0.23$

Statistical difference (p<0.05) within each column is indicated by different letters

PH% ranged from approximately 21.84 % to 47.70 %, with *Lb. delbrueckii* subsp. *lactis* LbL33 showing the highest proteolytic activity, followed by *Lb. delbrueckii* subsp. *delbrueckii* LbL39 (46.13 ± 1.31 %) and *Lb. delbrueckii* subsp. *lactis* LbL37 (46.04 ± 4.12 %). In contrast, *Lb. paracasei* AF43 and *Lb. plantarum* 297 exhibited the lowest values (21.84 ± 2.11 % and 24.53 ± 2.29 %, respectively).

The PH% estimation indicated a greater extent of protein hydrolysis compared to the DH% measured using the OPA method. It should be noted that the OPA method has certain limitations. Chen et al. (1979) reported that OPA reacts only weakly with cysteine, an amino acid present in high amounts in whey proteins (e.g., α-lactalbumin contains approximately 6.5 % cysteine), potentially leading to an underestimation of the degree of hydrolysis values (Spellman et al., 2003). *Lb. delbrueckii* subsp. *bulgaricus* and subsp. *lactis* are widely recognized for their ability to hydrolyze whey proteins and release peptides (Chourasia et al., 2024b; Hebert et al., 2008; Pescuma et al., 2008). However, despite the highest reduction of proteins, *Lb. delbrueckii* subsp. *lactis* LbL33 did not generate the highest concentration of low-MW peptides, possibly due to cellular uptake of the released peptides to meet physiological needs (Ter et al., 2024). In contrast, several strains of *Lb. delbrueckii* subsp. *delbrueckii* (e.g., LbL2, LbL12, and LbL14) showed a marked ability to accumulate small peptides (<3 kDa), with *Lb. delbrueckii* subsp. *delbrueckii* LbL2 reaching 1.05 ± 0.23 mg/mL, significantly higher than all other strains (p<0.05). To the best of our knowledge, no previous studies have confirmed peptide release by this species. Interestingly, *Lb. delbrueckii* subsp. *delbrueckii* LbL2 exhibited a slightly lower overall protein reduction (32.02 ± 1.28 %) compared to LbL33, yet was the most effective in generating peptides in the <3 kDa range. These findings suggest that *Lb. delbrueckii* subsp. *delbrueckii* LbL2 promotes a more controlled proteolysis, favoring the

accumulation of short peptides, which may be particularly advantageous for bioactivity purposes. Conversely, species such as *St. macedonicus*, *Lb. plantarum* and *Lb. paracasei* are known to be poorly proteolytic (Çetin et al., 2024; Pescuma et al., 2013; Savijoki et al., 2006); in fact, they produced comparatively lower peptide concentrations.

Peptide yields from the fermentation of whey were considerably lower than those obtainable through enzymatic hydrolysis, which can release over 90 % of peptides in just a few hours (Huang et al., 2010; Innocente et al., 2023). However, fermentation remains a promising approach because it allows the production of a wide range of qualitatively diverse peptides and enables *in situ* biofortification. In addition, fermentation offers a cost-effective and sustainable alternative to enzymatic hydrolysis, as it relies on the intrinsic proteolytic systems of microorganisms rather than expensive commercial enzymes (Tonini et al., 2024; Ulug et al., 2021).

2.3.3 Screening of Plant-derived LAB Isolates

A set of 43 strains obtained from plant-derived FFs was subjected to screening in PM, in which pea proteins served as the primary nitrogen source. Their proficiency in metabolizing pea proteins was assessed by recording turbidimetric growth curves in both PM and MRS broth, as described for dairy isolates in WM. Figure 2.4 presents example growth curves for two different strains: one exhibiting poor proliferation in PM, and another showing good growth in PM.

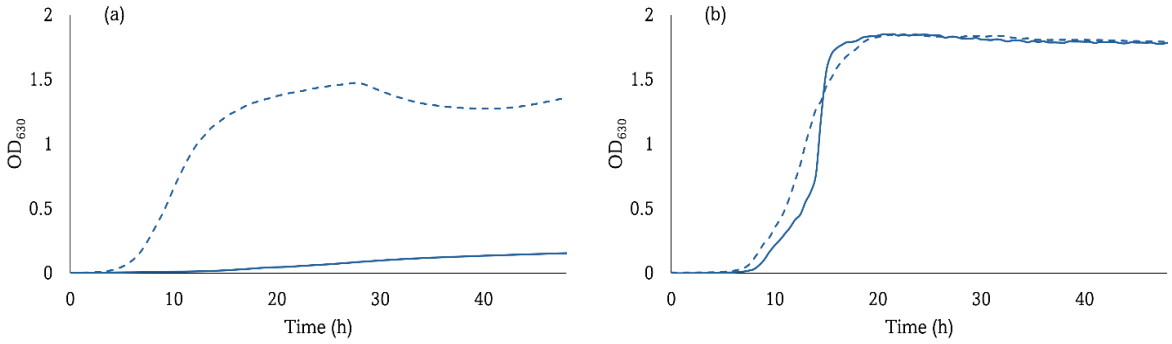

Figure 2.4 Growth curves of *Lb. pentosus* P1 (a) and *Lb. brevis* C2 (b) in MRS (dotted line) and pea medium (PM) (continuous line)

Lb. pentosus P1 showed no growth in PM, possibly due to limited ability to hydrolyze or assimilate pea proteins as a nitrogen source. In the present study, PM was supplemented with glucose to provide an available carbon source, yet certain strains failed to grow. This inability likely reflects the limited efficiency of their proteolytic systems in degrading pea proteins, whose amino acid profile lacks specific essential amino acids in free form (Chopin, 1993; Gorissen et al., 2018), thereby preventing them from meeting their nutritional requirements for growth. Although amino acids are essential for the growth of LAB, many species are auxotrophic for multiple amino acids. They must rely on their proteolytic system to release them from environmental protein sources

(Raveschot et al., 2018). However, not all strains possess an equally efficient proteolytic machinery, and proteolytic activity can be further modulated by environmental factors such as temperature, pH, and the physicochemical properties of the protein substrate (Cruz-Casas et al., 2021). In contrast, *Lb. brevis* C2 showed enhanced growth in PM, suggesting efficient proteolytic activity and adaptation to pea-protein substrates, potentially coupled with effective glucose utilization.

Overall, 28 of the 43 proteolytic isolates displayed growth in PM (Table 2.4). *Lb. brevis* was the most performing species, accounting for nine isolates, all originating from sauerkraut. *Lb. plantarum* followed with eight isolates from fermented *Brassica rapa* and sauerkraut. *P. pentosaceus* comprised five isolates from fermented *Brassica rapa* and sourdough. Other species included *Leuc. citreum*, *Leuc. pseudomesenteroides*, and *W. paramesenteroides*. In addition, single isolates of *Lb. paracasei* (K1A) and *P. acidilactici* (K4A), both from water kefir, also demonstrated growth in PM.

Table 2.4 LAB strains capable of growth in pea medium (PM)

Species	Strains
Lb. brevis (n=9)	C2, C31, C4, C5, C9, CRB, CRC, CRE, CRH
Lb. plantarum (n=8)	P4, P6, P9, P12, M2, CRA, CRD2, CRF
P. pentosaceus (n=5)	P2, P8, I1, I6, I7
Leuc. citreum (n=2)	C10, I10
Leuc. pseudomesenteroides (n=1)	I3
W. paramesenteroides (n=1)	I5
Lb. paracasei (n=1)	K1A
P. acidilactici (n=1)	K4A

All the samples capable of growing in pea protein showed increased DH% compared to the control PM (8.41 ± 0.55 %), with 25 of them being statistically higher (p<0.05) (Figure 2.5). The DH% across the samples ranged from 11.54 ± 2.08 % to 73.28 ± 3.83 %, indicating substantial variability in proteolytic activity. Among these, *Leuc. citreum* C10 and *Lb. brevis* C5 displayed the highest DH% values, reaching about 65 % and 73 %, respectively. The remaining isolates also demonstrated varying degrees of hydrolysis, many achieving moderate to high DH% levels, reflecting diverse but generally high proteolytic potential on pea protein. These results indicate that the ability to utilize pea proteins effectively is distributed across multiple LAB species.

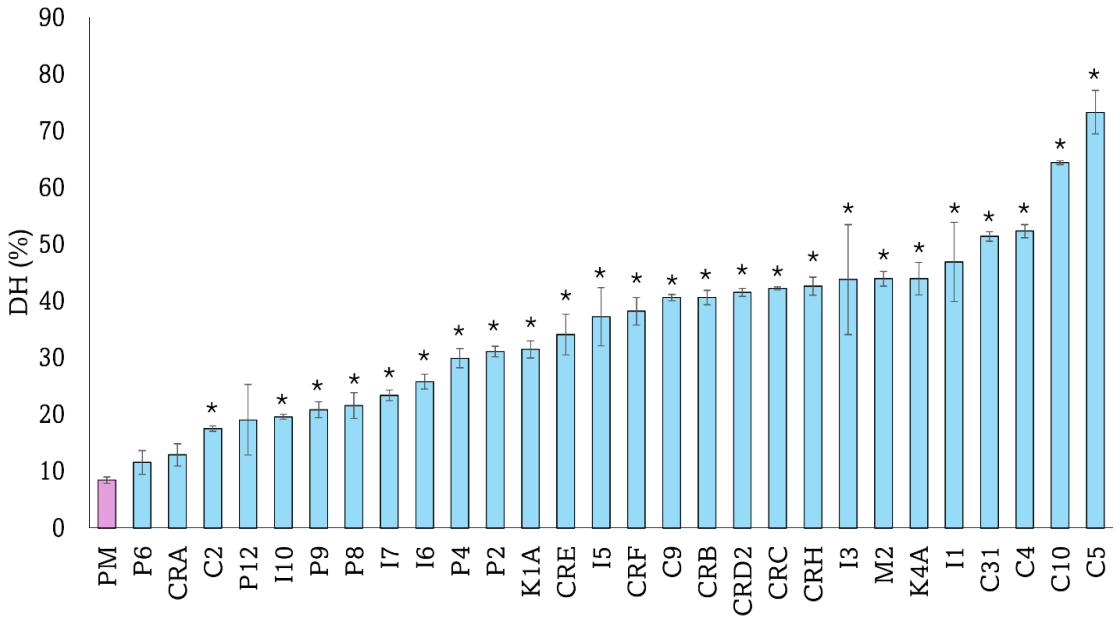

Figure 2.5 Results of the OPA analysis expressed as degree of hydrolysis (DH%). Data are expressed as mean ± standard deviation. Mean values marked with an asterisk are higher than the control (PM, shown in purple) (p<0.05)

All these species have been documented in the literature for their ability to grow on pea proteins or other legume-based substrates. *Lb. brevis* and *Lb. plantarum* have been shown to grow on and hydrolyze pea proteins, thereby improving the digestibility and reducing antinutritional factors (Çabuk et al., 2018; Kim et al., 2024a; Starzynska-Janiszewska & Stodolak, 2011). *Leuc. pseudomesenteroides*, *Lb. casei*, and *P. pentosaceus* have been reported to enhance pea flavor and physicochemical properties (Li et al., 2021a; Schindler et al., 2012; Sedó Molina et al., 2024b), while fermentation with *Lb. pentosus* and *Lc. lactis* has been shown to increase functional properties (e.g., antioxidant and cholesterol-lowering) of chickpea (Handa et al., 2022). Numerous studies have investigated the impact of these microorganisms on aroma, technological properties, and the improvement of pea protein digestibility. However, there is a lack of studies focusing on the use of these strains in fermentation to enhance the health-promoting properties of pea proteins, as well as studies exploring their proteolytic activity aimed at generating bioactive peptides (BPs).

As done for dairy isolates, peptide release after 72h-fermentation of PM was assessed through SE-HPLC. This approach is essential, as proteolysis does not always correlate with peptide accumulation, given that LAB may further degrade peptides into amino acids and utilize them for growth, thereby preventing their accumulation (Raveschot et al., 2018). Out of the 25 isolates screened, 7 were able to hydrolyze proteins and release peptides, as shown in Table 2.5. When comparing the peptide concentrations in the 6-3 kDa range, only two isolates, *Lb. brevis* C9 and *Leuc. pseudomesenteroides* I3 showed significantly higher values than the unfermented PM (0.09 ± 0.03 mg/mL), with *Leuc. pseudomesenteroides* I3 reaching the highest concentration (0.20 ± 0.02 mg/mL, p<0.05).

Table 2.5 Extent of protein hydrolysis (PH%) and concentration of peptides (mg/mL) in the 6–3 kDa and <3 kDa MW ranges released after 72-hour fermentation of PM. Values are presented as mean ± standard deviation

Sample	PH (%)	Peptide 6-3 kDa (mg/mL)	Peptide < 3 kDa (mg/mL)
PM	-	$0.09^{b} \pm 0.03$	$0.11^{b} \pm 0.01$
Lb. plantarum CRA	$70.41^{b} \pm 9.44$	$0.11^{b} \pm 0.01$	$0.22^{a} \pm 0.07$
Leuc. citreum C10	$69.33^{b} \pm 4.16$	$0.11^{b} \pm 0.03$	$0.22^{a} \pm 0.07$
Lb. plantarum M2	$68.39^{b} \pm 8.48$	$0.1^{b} \pm 0.00$	$0.23^{a} \pm 0.07$
Lb. brevis C4	$87.1^{a} \pm 7.63$	$0.1^{b} \pm 0.02$	$0.23^{a} \pm 0.07$
Lb. brevis C9	$83.54^{ab} \pm 0.66$	$0.14^{ab} \pm 0.06$	$0.31^{a} \pm 0.02$
Lb. brevis C31	$80.1^{ab} \pm 3.49$	$0.09^{b} \pm 0.00$	$0.30^{a} \pm 0.1$
Leuc. pseudomesenteroides I3	$87.84^{a} \pm 4.36$	$0.2^{a} \pm 0.02$	$0.35^{a} \pm 0.09$

Statistical difference (p<0.05) within each column is indicated by different letters

In contrast, for peptides smaller than 3 kDa, all the isolates produced significantly higher concentrations compared to PM (0.11 ± 0.01 mg/mL), with values ranging from 0.22 to 0.35 mg/mL. The highest concentration in this range was again observed for *Leuc. pseudomesenteroides* I3 (0.35 ± 0.09 mg/mL), followed by *Lb. brevis* C31 (0.30 ± 0.1 mg/mL) and *Lb. brevis* C9 (0.31 ± 0.02 mg/mL), albeit without statistical significance.

In parallel, the PH% was determined (Table 2.5). The highest PH% values were observed for *Leuc. pseudomesenteroides* I3 (87.84 ± 4.36 %) and *Lb. brevis* C4 (87.1± 7.63 %), followed by *Lb. brevis* C9 (83.54 ± 0.66 %) and *Lb. brevis* C31 (80.1 ± 3.49 %). These results suggest a correlation between higher protein hydrolysis and increased peptide accumulation, especially in the <3 kDa range. However, this relationship was not strictly linear across all samples, indicating that while extensive hydrolysis generally supports peptide accumulation, other strain-specific metabolic activities (e.g., peptide uptake and further degradation) may modulate the final peptide profile. Research on *Leuc. pseudomesenteroides* has primarily focused on its role as an exopolysaccharide producer (Perri et al., 2024; Xu et al., 2018), whereas its proteolytic activity has received comparatively little attention. In plant protein systems, this species has been studied for improving off-flavors and reducing antinutritional factors, with evidence that its proteolytic system can effectively degrade pea-based trypsin inhibitors (Sedó Molina et al., 2024a). Interestingly, its use in a pistachio-based beverage highlighted its potential to generate a wide variety of peptides with ACE and DPP-IV inhibitory properties, as well as antioxidant effects, yielding the highest amount and diversity of potentially BPs compared to the other strains tested in the study (Marulo et al., 2024). These results underscore the unexplored potential of this species as a source of functional protein fermentates from plant-based substrates. As for *Lb. brevis*, it has been associated with the production of bioactive compounds such as γ-aminobutyric acid after fermentation of pea proteins (Kim et al., 2025), as well as with improvements in protein digestibility and bioavailability through combined enzymatic treatment and fermentation (Kim et al., 2024a). In addition, *Lb. brevis* showed the ability to modify the molecular structure of pea proteins and enhance their functional properties, including solubility, emulsifying capacity, and foaming behavior across different pH levels (Kravchenko et al.,

2024). These findings indicate *Lb. brevis* as a promising candidate for tailoring the nutritional and techno-functional profile of pea protein. However, to date, no studies have reported its use to obtain pea protein fermentates with biological activities.

2.3.4 Fingerprint Typing of LAB Strains

Following the selection process, a total of 19 LAB strains were obtained, 7 plant-based and 12 dairy-derived, representing different species and showing promising features for further investigation. The genotypic diversity of isolates was analyzed through RAPD fingerprinting. For clarity, only profiles of the selected strains are shown (Figure 2.6). The obtained profiles, together with the corresponding dendrogram based on Pearson correlation, confirmed that each isolate represented a distinct strain, with primers D8635 and D11344 providing complementary resolution of genetic differences.

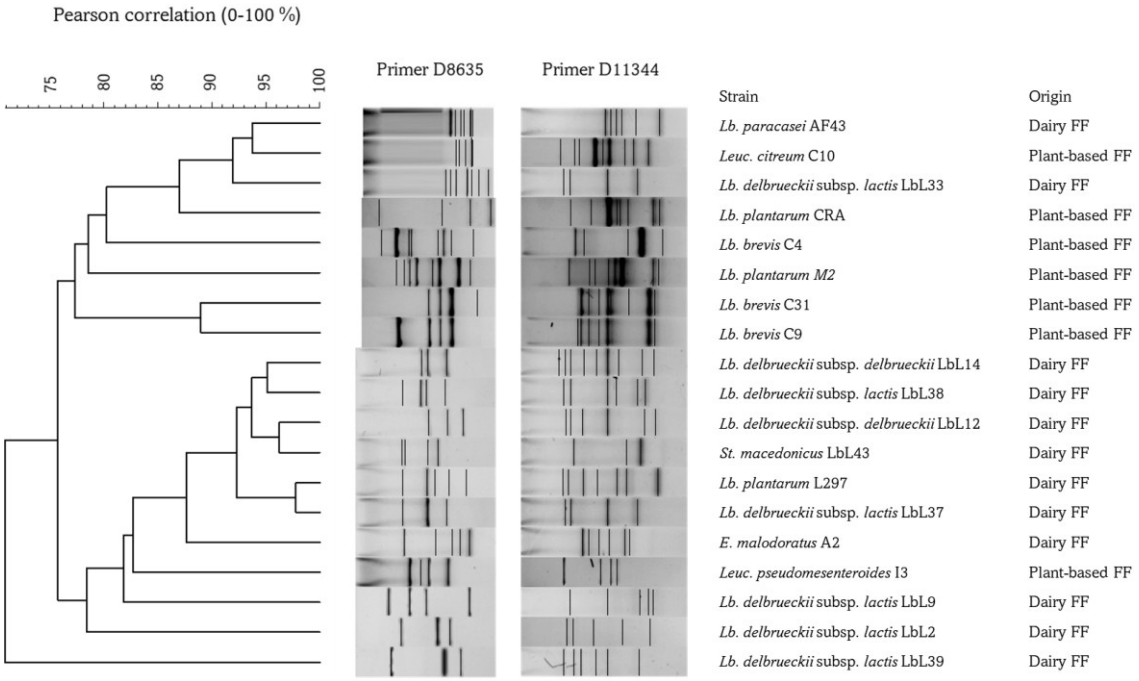

Figure 2.6 Dendrogram based on Pearson correlation (0–100 %) and RAPD-PCR fingerprinting profiles generated using primers D8635 and D11344 for 19 LAB strains isolated from dairy and plant-based FFs

Except for a few exceptions, strains clustered according to their source, with most dairy isolates grouping and plant-based isolates forming separate clusters, reflecting the influence of environmental origin on genetic similarity. Such differentiation likely reflects their metabolic adaptation to specific substrates and environmental conditions characteristic of dairy or plant-based ecosystems. This finding supports the idea that LAB from distinct environments may possess unique functional traits, making them valuable candidates for tailored biotechnological applications (O'Sullivan et al., 2009).

2.4 Conclusions

This chapter focused on the isolation, identification, and selection of LAB strains from different FFs. A total of 19 strains with unique genotypic profiles were selected, including 12 from dairy matrices, which were able to grow on whey proteins and hydrolyze them with the accumulation of peptides <3 kDa in the medium. The remaining 7 strains were isolated from plant-based fermented products. They were capable of growing on and hydrolyzing pea proteins, leading to the accumulation of peptides <3 kDa, and in some cases also in the 6-3 kDa range. These results highlight the potential of FFs as a valuable source of strains able to enhance the nutritional and functional value of alternative or by-product protein sources. Overall, the present findings provide a solid foundation for further investigation into the biological properties of the obtained fermentates.

Chapter 3: Unlocking The Biological Potential of Whey Proteins Through LAB Fermentation

3.1 Introduction

Whey, a major byproduct of the dairy industry, represents both an environmental challenge and a valuable nutritional resource. Every year, millions of tons of whey are generated worldwide. Due to its high biological oxygen demand, improper disposal can severely impact ecosystems (Chourasia et al., 2022b). At the same time, whey is a rich source of proteins, lactose, and bioactive molecules, making it an ideal candidate for biotechnological valorization. Beyond its use in animal feed or energy recovery, whey can be transformed into a wide array of functional ingredients and health-oriented products through innovative processing strategies (Goyal et al., 2023). Traditional approaches such as ultrafiltration and chromatographic separation have led to the development of whey protein concentrates and isolates with applications in sports nutrition and infant formula (Kelly, 2019). However, there has been relatively limited research on biological treatments, specifically microbial fermentation, aimed at enhancing whey for food applications.

Recent studies have highlighted the role of LAB in releasing bioactive peptides (BPs) from whey protein concentrate (WPC) obtained through cheese whey ultrafiltration. For instance, *Streptococcus thermophilus* has been employed to ferment WPC, yielding fermentates with angiotensin converting enzyme (ACE)-inhibitory, antidiabetic, and antioxidant activities. Similarly, *Lactobacillus helveticus* and *Lactobacillus acidophilus* strains have been shown to release antimicrobial peptides during growth in whey protein medium (Solieri et al.,2022). While starter cultures are well-known for their efficient proteolytic activity, much less is known about the potential of non-starter and non-commercial lactic acid bacteria (LAB) strains, especially those isolated from traditional raw-milk dairy products. These strains often exhibit unique proteolytic traits and metabolic behavior, which may favor the production of peptides with specific functional properties (Savijoki et al., 2006).

Furthermore, whey protein fermentates may also influence the gut microbiota and its metabolites. Emerging evidence suggests that whey proteins and derived peptides can act as prebiotic modulators, promoting the growth of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* while suppressing pathogenic species, contributing to gut homeostasis and protection against dysbiosis (Rackerby et al., 2024). These microbial modulations are accompanied by changes in the production of microbial metabolites, such as short-chain fatty acids (SCFAs), which play a crucial role in maintaining gut barrier integrity, regulating host immune responses, and contributing to energy metabolism (Boscaini et al., 2021)

Within this framework, the present study explored the capacity of twelve non-starter LAB strains to improve the health-promoting potential of whey medium (WM), as a model of whey-based fermented beverage. Fermentates were characterized for residual sugars, organic acids, free amino acids, and the extent of protein hydrolysis across different protein fractions. The functional

properties of the resulting fermentates were assessed in terms of antioxidant, ACE-inhibitory, and antimicrobial activities, as well as their influence on gut microbiota composition and metabolite profiles. By combining biochemical and microbiological characterization with bioactivity evaluation, this work provides new insights into the potential of LAB-fermented WM for sustainable food innovation and the development of functional fermented foods (FFs).

3.2 Materials and Methods

3.2.1 Materials

All culture media, including MRD, MRS broth, M17 broth, and Brain Heart Infusion (BHI) broth, along with lactose, glucose, and bacteriological agar, were procured from Oxoid (Milan, Italy). WPC containing 77 % protein was obtained from BulkTM (Colchester, UK). Chemicals and standards, including acetonitrile, BSA, β-Lg, α-La, CMP, aprotinin from bovine lung, C-peptide, L-glutathione oxidase, L-serine, DPPH, absolute ethanol, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®), 2,4,6-tris(2-pyridyl)-S-triazine (TPTZ), potassium chloride, hydrochloric acid, iron(III) chloride, angiotensin I-converting enzyme (ACE), hippuryl-L-histidyl-L-leucine, potassium-phosphate buffer, sodium chloride, sulfuric acid, methanol, N-α-(2,4-dinitro-5-fluorophenyl)-L-alanine amide (FDAA), sodium bicarbonate, acetonitrile, TFA, amino-acid kit (analytical standards), calcium chloride dihydrate sodium hydroxide, potassium chloride, magnesium chloride hexahydrate, potassium dihydrogen phosphate, ammonium carbonate, porcine pepsin (EC 3.4.23.1), bile extract from porcine (EC 232-369-0), pancreatin from porcine pancreas (EC 232-468-9; 8x USP), sodium hydroxide, Pefabloc SC, phosphate-buffered saline (PBS), tryptone, resazurin sodium salt, L-cysteine, sodium sulfide hydrate, acetic acid, propionic acid, butyric acid, formic acid, isovaleric acid, and isobutyric acid were all purchased from Merck (Darmstadt, Germany).

3.2.2 Bacterial Strains and Culture Conditions

The dairy strains isolated and selected in Chapter 2 (Table 3.1) were stored at -80 °C in MRS or M17 broth supplemented with 30 % glycerol (v/v). Cultures were reactivated in 1 mL of appropriate medium, then streaked onto agar plates and incubated for 48 h under anaerobic conditions.

Table 3.1 Bacterial strains and culture conditions

Strain	Culture conditions
Lacticaseibacillus paracasei AF43	MRS, 37 °C
Lactiplantibacillus plantarum 297	MRS, 37 °C
Streptococcus macedonicus LbL43	MRS, 37 °C
Lactobacillus delbrueckii subsp. *delbrueckii* LbL38	MRS, 37 °C
Lb. delbrueckii subsp. *bulgaricus* LbL9	MRS, 37 °C
Lb. delbrueckii subsp. *delbrueckii* LbL39	MRS, 37 °C
Lb. delbrueckii subsp. *lactis* LbL37	MRS, 37 °C
Lb. delbrueckii subsp. *lactis* LbL33	MRS, 37 °C
Enterococcus malodoratus A2	M17, 37 °C
Lb. delbrueckii subsp. *delbrueckii* LbL12	MRS, 30 °C
Lb. delbrueckii subsp. *delbrueckii* LbL14	MRS, 37 °C
Lb. delbrueckii subsp. *delbrueckii* LbL2	MRS, 30 °C

3.2.3 Characterization of Fermentates Derived from WM Fermentation

WM was prepared as described in Section 2.2.5.2. Overnight cultures were prepared by inoculating a single colony of each strain in 1 mL of MRS or M17. Each overnight culture was washed once in MRD, inoculated (1 % v/v) in WM, and incubated at 30 °C or 37°C for 72 h. Uninoculated WM served as a control. At the end of fermentation, supernatant was separated from cells by centrifugation (5,000 × g, 5 min, 4 °C), filtered (0.22 µm), and stored at -20°C for further analysis.

3.2.3.1 Bacterial Counts

Bacterial counts of fermentates were performed by serially diluting the samples and spread-plating onto MRS agar for lactobacilli, and M17 agar supplemented with 10 % (v/v) lactose for streptococci and enterococci. The samples were then incubated at 30 °C or 37 °C for 48 h under anaerobic conditions.

3.2.3.2 Determination of Sugars and Organic Acids

Sugars (lactose, glucose, and galactose) and organic acids (oxalic, citric, lactic, acetic, and formic) from WM and fermentates were quantified following the method proposed by Rossi et al. (2024) with slight modifications. In brief, samples were diluted 1:10 with 25 mM H_2SO_4 and homogenized for 5 min. The resulting suspension was centrifuged at 15,000 × g for 20 min at 4 °C. Separation of sugars and organic acids was achieved using an LC-4000 HPLC system (Jasco Europe, Lecco, Italy) equipped with an Aminex HPX-87H ion-exclusion column (300 mm x 7.8 mm x 9 µm; Bio-Rad, Hercules, CA), an AS-4050 autosampler, a 20 µL injection loop, and a column oven set at 65 °C. Chromatographic runs were performed at a flow rate of 0.7 mL/min with 25 mM H_2SO_4 as the mobile phase. Detection of organic acids was carried out with an MD-4010 PDA detector (Jasco Europe) at 210 nm, whereas sugars were monitored using an RI-4030 detector (Jasco Europe)

maintained at 40 °C. Quantitative analysis was performed by external calibration with standard solutions prepared at concentrations ranging from 0.001 to 100 mg/mL (R^2 = 0.99).

3.2.3.3 Determination of Free Amino Acids

Cell-free supernatants were prepared and processed following the method described by Gao et al. (2023), with minor modifications. Briefly, 1 mL of the sample was mixed with 9 mL of 80 % (v/v) methanol and subjected to ultrasonic treatment (45 kHz) at 30 °C for 60 min. The mixture was then centrifuged at 15,000 × g for 10 min, and the resulting supernatant was filtered through a 0.45 µm syringe filter. The filtrate was dried at 40 °C, and the residue was re-dissolved in 50 µL of milli-Q water.

For derivatization, 50 µL of either the standard solution or the extracted sample was transferred to a tube protected from light. Subsequently, 100 µL of FDAA solution (5 mg/mL in acetone) and 20 µL of $NaHCO_3$ solution (1 mol/L in milli-Q water) were added sequentially. The mixture was vortexed for 1 min and incubated at 40 °C for 90 min in the dark. After cooling to 25 °C, 10 µL of HCl (2 mol/L) and 420 µL of 40 % (v/v) aqueous acetonitrile were added in sequence. The sample was vortexed for 1 min and filtered through a 0.22 µm syringe filter.

HPLC analysis was performed using an LC-4000 system (Jasco Europe) equipped with an MD-4010 PDA detector. Separation was carried out on an Acclaim C18 column (4.6 mm × 250 mm, 5 µm; Thermo Fisher Scientific, Waltham, MA) maintained at 40 °C. For elution, the mobile phase A consisted of milli-Q water containing 5 % (v/v) acetonitrile, 1 % (v/v) methanol, and 0.05 % (v/v) TFA. Mobile phase B consisted of milli-Q water containing 60 % (v/v) acetonitrile, 1 % (v/v) methanol, and 0.05 % (v/v) TFA. The optimized gradient program was as follows: 0–22.5 min, 100 %–50 % A; 22.5–65.0 min, 50 %–18.5 % A; 65.0–75.0 min, 100 % A. The flow rate was set at 1.0 mL/min, the injection volume at 50 µL, and the detection wavelength at 280 nm. Quantification was performed by preparing standard solutions at different concentrations (0.0003-0.09 mg/mL) for external calibration (R^2 = 0.99). Results for each sample are expressed as relative abundance.

3.2.3.4 Extent of Protein Hydrolysis of Individual Whey-protein Fractions

The extent of protein hydrolysis and the MW distribution of peptides released during fermentation were estimated using SE-HPLC analysis, as described by Cui et al. (2022) and Innocente et al. (2023). A TSKgel 2000 SWXL 300mm x 7.8 mm column (Tosoh Bioscience, Griesheim, Germany) with a mobile phase consisting of water/acetonitrile/TFA (55/45/0.1, v/v/v) was used. The flow rate was 0.5 mL/min, and the column temperature was maintained at 30 °C. A 10-µL sample was injected into the LC-4000 system (Jasco Europe) equipped with a PDA detector set at 220 nm. BSA (66,463 Da), β-Lg (18,400 Da), CMP (6,800 Da), aprotinin (6,511 Da), C-peptide (3,183 Da), L-glutathione oxidised (612 Da), and L-Serine (106 Da) were run as standards. Data analysis was performed using the chromatography software ChromNAV2 (Jasco Europe). The extent of protein

hydrolysis (PH, %) was determined for individual whey protein fractions (e.g., BSA, α-La, β-Lg, and CMP) by monitoring the decrease in their specific peak areas. PH was expressed using the formula shown in Eq. 1:

$$PH\ (\%) = \frac{(Area\_WM - Area\_F)}{Area\_WM}\ x\ 100 \qquad \text{(Eq. 1)}$$

where Area_WM and Area_F represent the chromatographic peak areas of the unfermented WM and fermentates, respectively. These values refer to the area of a specific protein peak for evaluating the reduction of individual fractions.

3.2.4 Evaluation of the Biological Properties of the Fermentates

3.2.4.1 Radical Scavenging Activity

DPPH radical scavenging activity was determined using the method described by Cui et al. (2022), with some modifications. Briefly, 50 µL of each fermentation cell-free supernatant was mixed with 150 µL of fresh DPPH ethanolic solution (0.05 mg/mL) in triplicate wells of a U-bottomed 96-well polystyrene microplate (Corning Life Sciences, Corning, NY, USA). After 20 min of incubation in the dark, the absorbance was read at 517 nm at 25 °C using a Sunrise microplate reader (Tecan, Milan, Italy). The radical scavenging activity was computed as reported in Eq. 2:

$$Radical\ scavenging\ activity\ (\%) = \frac{Absi - Absf}{Absi}\ \text{x}\ 100 \qquad \text{(Eq. 2)}$$

where Abs_i and Abs_f are the absorbances at the beginning and the end of the assay, respectively.

3.2.4.2 Ferric Reducing Antioxidant Power

FRAP of fermentation supernatants was determined using the method described by Benzie & Strain (1996), with some modifications. FRAP reagent was obtained by mixing KCl/HCl buffer (0.2 M; pH 2.2), 10 mM TPTZ, and a 20 mM FeCl3 solution in a 10:1:1 ratio. 100 µL of sample and 100 µL of FRAP reagent were placed in U-bottomed microplate wells. For each sample, three replicates were analyzed. The absorbance reading was carried out after 40 min using a Sunrise microplate reader (Tecan) at 593 nm at 37 °C. The FRAP (%) was calculated according to Eq. 3:

$$FRAP\ (\%) = \frac{Absf - Absi}{Absf} \qquad \text{(Eq. 3)}$$

where Abs_i and Abs_f were the absorbances at the beginning and the end of the assay, respectively.

3.2.4.3 ACE-inhibitory Activity

ACE-inhibitory activity was measured by HPLC following the method proposed by Cushman & Cheung (1971), with some modifications. Hippuryl-L-histidyl-L-leucine (5 mM) and ACE (0.1 U/mL) were dissolved in 0.1 M potassium phosphate buffer (pH 8.3) containing 0.3 M NaCl. The

assay was performed by mixing 120 µL of hippuryl-L-histidyl-L-leucine solution with 30 µL of each fermentation cell-free supernatant or milliQ water as a control. After 10 min of incubation at 37 °C, the reaction was started by adding 30 µL of ACE solution. The samples were incubated at 37 °C for 60 min under shaking, and then 150 µL of HCl (1 M) was added to stop the reaction. Samples were filtered through a 0.45 µm nylon syringe filter. Hippuric acid released by ACE was analyzed by injecting 6 µL of the sample in a Water Nova-Pak C18 column (4.6x150 mm, 4 µm) connected to an LC-4000 system (Jasco Europe) and detected at 228 nm. Elution was performed at a flow rate of 0.8 mL/min. The mobile phase comprised 0.1 % (v/v) TFA in milliQ water (solvent A) and 0.1 % (v/v) TFA in acetonitrile (solvent B), with a 10-60 % B gradient for 10 min, then returned to 10 % B in 2 min and followed by isocratic elution for 3 min. The percentage inhibition was calculated following Eq. 4:

$$ACE\ inhibitory\ activity\ (\%) = \frac{(Area\_control - Area\_sample)}{Area\_control} x\ 100 \qquad \text{(Eq. 4)}$$

where Area_sample is the chromatographic peak area of HA in the presence of the fermentates, and Area_control is the area obtained in the absence of the fermentate.

3.2.4.4 Antimicrobial Activity

The antimicrobial activity of each fermentation cell-free supernatant was tested against *Staphylococcus aureus*, *Salmonella enterica*, *Escherichia coli*, *Listeria monocytogenes*, *Enterococcus,* and *Pseudomonas fluorescens* by turbidimetric growth curves. For each microbial target, pools of three different strains were tested. *Staph. aureus* Di4A_226, *Staph. aureus* Di4A_CAR1-N, *Staph. aureus* DSMZ 2569, *Salm. enterica* spp. *arizonae* DSMZ 9386, *Salm. enterica* Di4A_#40, *Salm. enterica* Di4A_#52, *E. coli* Di4A_8048, *E. coli* Di4A_TOL, *E. coli* Di4A_UD, *L. monocytogenes* Scott A, *L. monocytogenes* Di4A_248, *L. monocytogenes* DSMZ 7644, *Ent. faecium* DMSZ 2146, *Ent. faecium* Di4A_TO, *Ent. durans* Di4A_173, *Ps. fluorescens* Di4A_LS1, *Ps. fluorescens* Di4A_LS3, and *Ps. fluorescens* DIAL22 from the bacterial collection of the Department of AgriFood, Environmental and Animal Sciences (Di4A) of the University of Udine were stored at −80 °C in BHI added with 30 % (v/v) glycerol. An overnight culture was prepared for each target strain by inoculating 1 mL of BHI with a loopful of the cryopreserved stock culture, followed by incubation at 37 °C overnight. Cells were recovered by centrifugation at 13,000 × g for 5 min in a Mikro 20 centrifuge (Hettich Italia s.r.l., Milan, Italy) and washed twice with MRD. Microbial pools were created by combining strains belonging to the same genus. Duplicate wells of a U-bottomed 96-well polystyrene microplate (Corning Life Sciences) were filled with 100 µL of BHI broth 2x and 100 µL of each cell-free supernatant (final pH 6.6 ± 0.2). Each well was inoculated with 10 µL of each microbial pool (final viability about $5x10^4$ cfu/mL). Wells containing inoculated BHI broth were used as a control (BHI), and wells containing non-inoculated medium as a blank. Microbial growth was monitored at 30 °C (for *Pseudomonas*

spp.) or 37 °C for 48 h using a Sunrise microplate reader (Tecan). The optical density at 630 nm (OD_{630}) was recorded every 30 min, preceded by 5 s shaking. Growth curve data were fitted using OriginLab 2021 (Northampton, UK) by the re-parametrised Gompertz equation (Zwietering et al., 1990) as reported in Eq. 5:

$$y = Aexp\left\{-exp\left[\frac{\mu_{max}*e}{A}(\lambda - t) + 1\right]\right\} \qquad \text{(Eq. 5)}$$

where t is time (h); y is response (log OD); A (amplitude) is the upper asymptote; μ_{max} is the maximum specific growth rate (log OD/h); and λ is the lag time (h). The parameters λ, μ_{max}, and A were estimated from the fitted model.

3.2.5 Effect on Gut Microbiota and Its Metabolites

3.2.5.1 *In vitro* Gastrointestinal Digestion

In vitro gastrointestinal digestion was simulated on WM and its fermentates in their entirety, including microbial cells, using the static INFOGEST protocol described by Brodkorb et al. (2019). Simulated salivary (SSF), gastric (SGF), and intestinal (SIF) fluids were freshly prepared, stored at 4 °C, and equilibrated at 37 °C before use. To simulate the oral phase, 1 mL of sample was mixed with 800 µL of SSF and 5 µL of 0.3 M $CaCl_2{\cdot}(H_2O)_2$. Distilled water was added to adjust the volume to 2 mL, and the mixture was gently stirred at 37 °C for 2 min. The gastric phase was initiated by supplementing the sample with 1.6 mL SGF, 133 µL of pepsin solution (dissolved in water to provide 2000 U/mL in the final digestion system), and 1 µL of 0.3 M $CaCl_2{\cdot}(H_2O)_2$. The pH was adjusted to 3 using 6 M HCl, and the volume was brought to 4 mL with distilled water. The sample was maintained at 37 °C under continuous agitation for 2 h. After 120 min, the intestinal phase was initiated by adding 1.6 mL SIF, 8 µL $CaCl_2{\cdot}(H_2O)_2$, 0.6 mL of bile solution (freshly prepared in SIF), and 1 mL of pancreatin solution (in SIF, yielding 100 U/mL in the final mixture). The pH was immediately raised to 6.5-7.0 and, if necessary, further adjusted to 7.0 with 1 M NaOH. MilliQ water was then added to reach a final volume of 8 mL. Incubation continued at 37 °C with stirring for an additional 2 h. At the end of the gastric and intestinal phases, gastric digesta were cooled on ice, while intestinal digesta were supplemented with Pefabloc® (5 mM) to stop enzymatic activity. Bacterial enumeration was performed before digestion (UND) and on both gastric (G) and intestinal (I) samples as detailed in Section 3.2.3.1. All digesta from the intestinal phase were centrifuged with an Avanti™ J-25 centrifuge (Beckman Coulter, Brea, CA, USA) at 11,000 × g for 10 min at 4 °C to separate pellet and supernatant fractions. Blank digestions contained all enzymes and simulated fluids, with distilled water replacing the test sample. Each *in vitro* digestion was carried out in duplicate.

3.2.5.2 *In vitro* Colonic Fermentation

The *in vitro* fermentation protocol was adapted from Pérez-Burillo et al. (2021). Fecal material was obtained from six healthy adult volunteers (three males and three females, aged 20-35 years) who followed an omnivorous diet and had no history of gastrointestinal disorders (e.g., celiac disease, Crohn's disease, diverticulitis). None had taken antibiotics, antacids, or probiotics within the previous six months, and all tested negative for SARS-CoV-2 at the time of recruitment. Written informed consent was obtained from each participant before sample collection. The study was approved by the Institutional Review Board of the Department of AgriFood, Environmental, and Animal Sciences, University of Udine, on 06/02/2024. Fecal samples were collected in sterile containers under anaerobic conditions, stored at refrigerated temperature, and processed within two hours. Samples were pooled, homogenized with glycerol (20 % w/v) in a 1:1 ratio, and stored at −80 °C until analysis. For preparation of the fecal slurry, the thawed pooled sample was centrifuged (4,000 × g, 10 min, 4 °C), and the resulting pellet was resuspended in PBS (0.1 M, pH 7) to a final concentration of 32 % (w/v). The suspension was vortexed for 1 min and centrifuged at 550 × g for 5 min. The supernatant was collected and used as the fermentation inoculum. The fermentation medium consisted of 50 mL of tryptone solution (15 g/L, pH 7; sterilized at 121 °C for 15 min), 62.5 µL of resazurin sodium salt solution (1 mg/mL; sterilized at 121 °C for 15 min), and 2.5 mL of reductive solution. The reductive solution was prepared by dissolving 312 mg of L-cysteine and 312 mg of sodium sulfide hydrate in 2 mL of NaOH (1 M), adjusting the volume to 50 mL with Milli-Q water, and sterilizing through a 0.22 µm filter. The medium was pre-reduced in an anaerobic chamber before use. Each fermentation mixture contained 750 µL of fermentation medium, 200 µL of fecal slurry, and 50 mg of the digested sample, assembled under anaerobic conditions. Fermentations were conducted at 37 °C for 24 h under anaerobic conditions with gentle agitation (20 rpm) in the dark. All trials were performed in duplicate. Fermentation blanks were prepared using Milli-Q water in place of the sample. Following fermentation, samples were centrifuged (16,000 × g, 2 min, 4 °C). The pellet was processed for metagenomic 16S rRNA sequencing, while the 0.22 µm-filtered supernatant was reserved for SCFA and branched-chain fatty acid (BCFA) analysis.

3.2.5.3 DNA Extraction, Library Preparation, and Sequencing

Fecal pellets were centrifuged at 16,000 × g for 2 min at 4 °C, diluted to 10 % (w/v), and subjected to DNA extraction using the Mag-Bind® Universal Pathogen 96 Kit (Omega Biotek, Winooski, VT, USA) following the manufacturer's instructions. DNA extracts were subsequently processed for library preparation according to the Illumina 16S Metagenomic Sequencing Library Preparation protocol. The V3–V4 hypervariable region of the 16S rRNA gene was amplified using primers 341F (CTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC). Libraries were

sequenced on the Aviti platform (Element Biosciences, San Diego, CA, USA) using 300-bp paired-end mode.

3.2.5.4 Bioinformatic Analysis

Sequence processing and taxonomic assignment were carried out as previously described by Guazzini et al. (2025), using DADA2 (Callahan et al., 2016). Briefly, primers were removed, and reads were trimmed at 17 bp at the 3' end and at 21bp of the 5' end based on error profiles. Paired reads were merged, and amplicon sequence variants were identified and classified using the Silva database (https://www.arb-silva.de/). Outputs were obtained as both raw read counts and relative abundances, expressed as percentages of total reads. Alpha-diversity metrics, including the observed number of taxa, Shannon index, and Simpson index, were calculated from raw counts using the vegan package in R (https://CRAN.R-project.org/package=vegan) (v 4.3.0, The R Foundation for Statistical Computing, Vienna, Austria).

3.2.5.5 Determination of Short- and Branched-Chain Fatty Acids

SCFA and BCFAs were quantified using the method of Ashaolu et al. (2019), with minor modifications. HPLC analyses were performed on a LC-4000 system (Jasco Europe) equipped with an autosampler, a 20 µL injection loop, and a photodiode array. Separation was carried out using an Aminex HPX-87H ion-exclusion column (300x7.8 mm; Bio-Rad, Richmond, CA, USA) with 0.005 M H_2SO_4 as the mobile phase, delivered at a flow rate of 0.6 mL/min. The column temperature was set at 35 °C, and each chromatographic run lasted 45 min. Compounds were detected by UV absorbance at 210 nm. SCFA and BCFA concentrations were quantified from peak areas using external calibration curves (R^2 = 0.99) prepared with standard solutions ranging from 0 to 50 mM.

3.2.6 Statistical Analysis

All trials were carried out at least in duplicate, and values were expressed as the means ± standard deviation. Statistical analysis was performed using R v 4.3.0 (The R Foundation for Statistical Computing). Bartlett's test was used to check the homogeneity of variance. The difference between means was calculated using a t-test and one-way ANOVA, and the Tukey test was used to determine statistically significant differences among means (p<0.05). Principal component analysis (PCA) and hierarchical clustering were performed using the vegan package in R v 4.3.0 (The R Foundation for Statistical Computing). Correlations between the relative abundance of microbial taxa and the concentrations of SCFAs and BCFAs, residual sugars and organic acids, amino acids, and peptides were explored using Spearman's correlation coefficients and visualised with the *corrplot* package in R (https://github.com/taiyun/corrplot). Only taxa and variables with <50 % missing data were included in the correlation analyses.

3.3 Results and Discussion

3.3.1 Characterization of Fermentates Derived from WM Fermentation

WM was subjected to fermentation by twelve different strains of LAB, previously isolated from dairy products and selected for their proteolytic metabolism and ability to grow in the presence of whey protein. All the strains showed to grow in WM, reaching approximately 6-8 log cfu/mL (data not shown). WM is a protein- and lactose-rich substrate, providing both nitrogen and carbon sources that influence microbial growth, fermentation dynamics, and the nutritional quality of the final product. Evaluating both sugar consumption and amino acid profiles is essential to understanding strain-specific metabolic activity and its impact on product composition.

Some LAB can ferment lactose, the main sugar present in dairy substrates. After internalization inside the cell, it is first hydrolyzed by β-galactosidase into glucose and galactose. While glucose is rapidly metabolized and reduced to lactic acid or other organic acids, the fate of galactose is more variable. In the Leloir pathway, galactose is converted into glucose-6-phosphate, whereas in the tagatose-6-phosphate pathway, it is transformed into triose phosphates. Both processes ultimately feed into glycolysis. However, many strains are galactose-negative and tend to export galactose rather than consume it (Iskandar et al., 2019). Therefore, lactose metabolism in LAB involves a strain-dependent balance between efficient glucose fermentation and variable galactose utilization, resulting in characteristic profiles of sugar consumption and organic acid production. For this reason, it is crucial to evaluate both the residual sugars and the organic acids generated during fermentation, as these parameters provide insight into the metabolic activity of different strains. In Table 3.2, the concentrations of residual sugars and organic acids are reported for WM and fermentates obtained with different LAB, allowing comparison of their metabolic performance. WM contained lactose (54.77 ± 1.19 mg/mL) as the sole carbohydrate before fermentation. Following fermentation, lactose concentrations decreased to varying extents depending on the strain (p<0.05). Specific LAB, such as *Lb. paracasei* AF43 and *Lb. delbrueckii* strains efficiently utilized lactose. *Lb. paracasei*, *Lb. delbrueckii* (subsp. *bulgaricus*, *lactis*, and *delbrueckii*), and *St. macedonicus* are generally lactose-positive, possessing both lactose transport systems and β-galactosidase that facilitate lactose hydrolysis (Bintsis, 2018b; Papadimitriou et al., 2014). In contrast, *Lb. plantarum* 297 exhibited little lactose consumption. In *Lb. plantarum*, the efficiency of lactose uptake and the level of β-galactosidase expression vary among strains and depend on environmental conditions (Xu et al., 2022). Galactose was not detected in WM; however, it accumulated in some fermentates, reflecting differences in its metabolism across LAB strains. Several *Lb. delbrueckii* are reported to export galactose rather than metabolizing it (Iskandar et al., 2019), which explains why almost all *Lb. delbrueckii* strains accumulated galactose to different extents (0.42-1.60 mg/mL).

Table 3.2 Sugars and organic acids (mg/mL; mean ± standard deviation) detected in whey medium (WM) and fermentates

Strain	Lactose	Galactose	Lactic acid	Acetic acid	Formic acid	Oxalic acid	Citric acid
WM	$54.77^{a} \pm 1.19$	n.d.	n.d.	$0.17^{e} \pm 0.01$	$0.14^{c} \pm 0.00$	$0.23^{d} \pm 0.01$	$0.02^{d} \pm 0.00$
Lb. paracasei AF43	$47.45^{d} \pm 0.05$	n.d.	$3.69^{a} \pm 0.01$	$0.27^{cd} \pm 0.01$	$0.18^{ab} \pm 0.01$	$0.31^{c} \pm 0.00$	$0.03^{c} \pm 0.00$
Lb. plantarum 297	$54.01^{a} \pm 0.09$	n.d.	$1.77^{d} \pm 0.01$	$0.26^{cd} \pm 0.01$	n.d.	$0.31^{c} \pm 0.00$	$0.03^{c} \pm 0.00$
St. macedonicus LbL43	$50.98^{b} \pm 0.08$	n.d.	$0.85^{i} \pm 0.07$	$0.53^{a} \pm 0.03$	$0.20^{a} \pm 0.00$	$0.30^{c} \pm 0.02$	$0.06^{b} \pm 0.01$
Lb. delbrueckii subsp. *delbrueckii* LbL38	$52.56^{ab} \pm 0.03$	n.d.	$0.80^{i} \pm 0.01$	$0.40^{b} \pm 0.01$	$0.16^{b} \pm 0.00$	$0.31^{c} \pm 0.00$	$0.06^{b} \pm 0.00$
Lb. delbrueckii subsp. *bulgaricus* LbL9	$53.81^{ab} \pm 0.35$	$1.28^{b} \pm 0.01$	$1.56^{f} \pm 0.02$	$0.23^{d} \pm 0.03$	$0.16^{b} \pm 0.01$	$0.35^{b} \pm 0.01$	$0.07^{b} \pm 0.00$
Lb. delbrueckii subsp. *delbrueckii* LbL39	$52.31^{ab} \pm 0.06$	$0.42^{f} \pm 0.01$	$1.13^{h} \pm 0.01$	$0.29^{c} \pm 0.01$	$0.18^{ab} \pm 0.01$	$0.34^{b} \pm 0.00$	$0.07^{b} \pm 0.00$
Lb. delbrueckii subsp. *lactis* LbL37	$49.69^{bc} \pm 0.06$	$1.32^{b} \pm 0.07$	$1.92^{c} \pm 0.00$	$0.30^{c} \pm 0.01$	$0.18^{ab} \pm 0.00$	$0.33^{bc} \pm 0.01$	$0.06^{b} \pm 0.00$
Lb. delbrueckii subsp. *lactis* LbL33	$53.22^{ab} \pm 0.03$	$1.14^{c} \pm 0.01$	$1.64^{e} \pm 0.04$	$0.26^{cd} \pm 0.03$	$0.16^{b} \pm 0.01$	$0.33^{bc} \pm 0.00$	$0.06^{b} \pm 0.01$
Ent. malodoratus A2	$53.14^{ab} \pm 0.04$	n.d.	$1.42^{g} \pm 0.01$	$0.23^{d} \pm 0.01$	$0.16^{b} \pm 0.00$	$0.31^{c} \pm 0.00$	$0.03^{c} \pm 0.00$
Lb. delbrueckii subsp. *delbrueckii* LbL12	$48.37^{c} \pm 0.05$	$1.60^{a} \pm 0.03$	$2.15^{b} \pm 0.01$	$0.27^{cd} \pm 0.02$	$0.19^{ab} \pm 0.01$	$0.36^{b} \pm 0.01$	$0.07^{b} \pm 0.00$
Lb. delbrueckii subsp. *delbrueckii* LbL14	$48.41^{c} \pm 0.05$	$1.03^{d} \pm 0.02$	$1.92^{c} \pm 0.01$	$0.25^{cd} \pm 0.00$	$0.18^{ab} \pm 0.00$	$0.35^{b} \pm 0.02$	$0.06^{b} \pm 0.00$
Lb. delbrueckii subsp. *delbrueckii* LbL2	$50.08^{b} \pm 0.04$	$0.81^{e} \pm 0.00$	$1.38^{f} \pm 0.03$	$0.26^{cd} \pm 0.04$	$0.20^{a} \pm 0.02$	$0.43^{a} \pm 0.02$	$0.09^{a} \pm 0.00$

Statistical difference ($p<0.05$) within each column is indicated by different letters
n.d. not detected

During carbohydrate metabolism in LAB, glucose is converted to pyruvate as the central metabolic intermediate. In homofermentative LAB, pyruvate is reduced almost exclusively to lactic acid via lactate dehydrogenase, a reaction that ensures regeneration of NAD^+ and maintenance of redox balance (Garrigues et al., 2001). Indeed, all strains produced lactic acid, whereas it was absent in WM, confirming active carbohydrate metabolism. Lactic acid was the predominant product, ranging from 0.80 ± 0.01 mg/mL (*Lb. delbrueckii* subsp. *delbrueckii* LbL38) to 3.69 ± 0.01 mg/mL (*Lb. paracasei* AF43). Interestingly, *Lb. plantarum* 297 produced a considerable amount of lactic acid (1.77 ± 0.01 mg/mL) despite little consumption of lactose. This is likely because it utilized galactose released from lactose hydrolysis, which was not detected in the medium, and possibly also amino acids or peptides as alternative energy sources. Higher lactose consumption generally corresponded to higher lactic acid production: for instance, *Lb. paracasei* AF43 combined strong lactose hydrolysis with the highest lactic acid output. In addition to lactic acid, smaller amounts of secondary acids were detected.

Oxalic acid (0.30-0.43 mg/mL) and citric acid (0.03-0.09 mg/mL) remained minor metabolites across strains. Both oxalic and citric acids are naturally present in milk and its derivatives, as a result of the bovine diet and intrinsic milk metabolism (Walstra et al., 2005). Formic acid (0.16-0.20 mg/mL) and acetic acid (0.23-0.53 mg/mL) were more strain-specific, reflecting side routes of pyruvate metabolism. Actually, pyruvate can also be metabolized through alternative routes depending on the species, strain, and environmental conditions. These include its conversion into acetic acid or other organic acids (e.g., formic acid) and volatiles (Gänzle, 2015). Interestingly, *St. macedonicus* LbL43 and *Lb. delbrueckii* subsp. *delbrueckii* LbL38 produced the highest concentration of acetic acid (0.53 ± 0.03 and 0.40 ± 0.01 mg/mL, respectively), together with the lowest amount of lactic acid. This pattern suggests a shift towards a mixed-acid fermentation profile under the tested conditions, despite both species being generally described in the literature as homofermentative. Such metabolic flexibility enables LAB to adapt to variations in substrate availability and redox constraints, occasionally resulting in mixed-acid fermentation profiles, even in strains generally classified as homofermentative (Bintsis, 2018b; De Vuyst & Tsakalidou, 2008; Hebert et al., 2013).

Whey proteins are a rich source of essential amino acids, in particular branched-chain amino acids (leucine, isoleucine, and valine). Whey also contains significant amounts of sulfur-containing amino acids such as cystine and methionine (Olvera-Rosales et al., 2023). Assessing the free amino acid profile after LAB fermentation is vital to determine how microbial activity alters the availability of both essential and non-essential amino acids, ultimately affecting the nutritional and functional properties of whey-based products. Table 3.3 reports the relative abundance (%) of amino acids detected in WM and in the corresponding fermentates.

Table 3.3 Relative abundance (%; mean ± standard deviation) of amino acids detected in whey medium (WM) and fermentates

Strain	Histidine	Threonine	Cystine	Alanine	Proline	Tyrosine	Phenylalanine	Leucine	Cysteine	Lysine
WM	n.d.	$8.3^{a} \pm 2.38$	$4.67^{a} \pm 1.22$	n.d.	$22.85^{bc} \pm 8.04$	$48.37^{a} \pm 11.01$	n.d.	$2.77^{a} \pm 0.29$	$1.96^{ab} \pm 0.23$	$11.03^{bc} \pm 3.52$
Lb. paracasei AF43	n.d.	$6.86^{a} \pm 1.85$	$4.55^{a} \pm 1.33$	n.d.	$26.88^{bc} \pm 7.71$	$30.31^{ab} \pm 6.16$	n.d.	$3.22^{a} \pm 1.20$	$2.88^{ab} \pm 1.49$	$25.29^{a} \pm 10.2$
Lb. plantarum 297	n.d.	$6.95^{a} \pm 3.13$	$4.94^{a} \pm 1.14$	n.d.	$36.53^{b} \pm 8.94$	$44.19^{a} \pm 7.92$	n.d.	$2.57^{a} \pm 0.12$	$1.72^{ab} \pm 0.10$	$3.10^{c} \pm 2.64$
St. macedonicus LbL43	n.d.	$6.6^{a} \pm 1.34$	$5.07^{a} \pm 0.97$	n.d.	$37.37^{b} \pm 9.46$	$43.69^{a} \pm 5.47$	n.d.	$2.16^{a} \pm 0.70$	$1.41^{ab} \pm 0.57$	$3.70^{c} \pm 0.79$
Lb. delbrueckii subsp. *delbrueckii* LbL38	$0.05^{b} \pm 0.03$	$7.56^{a} \pm 1.99$	$4.67^{a} \pm 1.23$	n.d.	$36.86^{b} \pm 6.46$	$43.78^{a} \pm 8.49$	n.d.	$1.66^{a} \pm 0.21$	$0.85^{b} \pm 0.27$	$4.56^{c} \pm 0.51$
Lb. delbrueckii subsp. *bulgaricus* LbL9	n.d.	$9.49^{a} \pm 2.43$	$7.04^{a} \pm 2.07$	$0.25^{a} \pm 0.16$	$18.74^{c} \pm 4.96$	$32.38^{ab} \pm 7.19$	n.d.	$2.87^{a} \pm 0.44$	$1.95^{ab} \pm 0.42$	$27.28^{a} \pm 6.52$
Lb. delbrueckii subsp. *delbrueckii* LbL39	n.d.	$3.96^{a} \pm 0.85$	$4.04^{a} \pm 3.21$	n.d.	$55.76^{a} \pm 3.13$	$21.59^{b} \pm 2.88$	n.d.	$2.49^{a} \pm 1.12$	$3.35^{a} \pm 2.26$	$8.75^{bc} \pm 3.90$
Lb. delbrueckii subsp. *lactis* LbL37	n.d.	$5.19^{a} \pm 3.00$	$5.22^{a} \pm 1.11$	n.d.	$39.41^{b} \pm 12.2$	$42.83^{ab} \pm 6.02$	$0.51^{a} \pm 0.05$	$2.21^{a} \pm 0.50$	$1.39^{ab} \pm 0.44$	$3.22^{c} \pm 0.76$
Lb. delbrueckii subsp. *lactis* LbL33	$0.49^{a} \pm 0.2$	$6.62^{a} \pm 1.56$	$4.76^{a} \pm 1.21$	n.d.	$35.45^{b} \pm 13.00$	$46.88^{a} \pm 8.82$	n.d.	$1.98^{a} \pm 0.39$	$1.08^{ab} \pm 0.31$	$2.76^{c} \pm 0.86$
Ent. malodoratus A2	n.d.	$7.92^{a} \pm 2.33$	$5.08^{a} \pm 1.39$	n.d.	$19.62^{bc} \pm 12.01$	$43.87^{a} \pm 10.05$	n.d.	$3.10^{a} \pm 0.37$	$2.28^{ab} \pm 0.30$	$18.05^{ab} \pm 3.60$
Lb. delbrueckii subsp. *delbrueckii* LbL12	n.d.	$6.39^{a} \pm 1.29$	$4.43^{a} \pm 1.16$	n.d.	$38.16^{b} \pm 7.61$	$36.04^{a} \pm 6.16$	n.d.	$1.80^{a} \pm 0.10$	$1.33^{ab} \pm 0.05$	$11.75^{bc} \pm 1.89$
Lb. delbrueckii subsp. *delbrueckii* LbL14	n.d.	$5.51^{a} \pm 1.47$	$4.74^{a} \pm 2.35$	n.d.	$39.05^{b} \pm 6.86$	$30.45^{ab} \pm 6.18$	n.d.	$2.46^{a} \pm 0.54$	$2.13^{ab} \pm 0.68$	$15.57^{ac} \pm 5.20$
Lb. delbrueckii subsp. *delbrueckii* LbL2	n.d.	$4.27^{a} \pm 0.60$	$4.37^{a} \pm 1.49$	n.d.	$47.38^{ab} \pm 7.46$	$30.4^{ab} \pm 6.64$	n.d.	$1.96^{a} \pm 0.41$	$1.66^{ab} \pm 0.56$	$9.94^{bc} \pm 4.01$

Statistical difference ($p<0.05$) within each column is indicated by different letters

n.d. not detected

In unfermented WM, tyrosine (48.37 ± 11.01 %), proline (22.85 ± 8.04 %), and lysine (11.03 ± 3.52 %) were the most abundant amino acids, while histidine, alanine, and cysteine were present at lower levels, and phenylalanine was not detected. Cystine (4.67 ± 1.22 %) was also a relevant component, consistent with the high content of sulfur amino acids in whey proteins. Some LAB strains increased the abundance of proline, as observed in *Lb. delbrueckii* subsp. *delbrueckii* LbL39 (over 30 % increase) and *Lb. delbrueckii* subsp. *delbrueckii* LbL2 (almost 25 %), likely reflecting limited catabolism of this residue. Tyrosine was not generally affected by fermentation, although in some fermentations it decreased, for example, in *Lb. delbrueckii* subsp. *delbrueckii* LbL39, suggesting microbial consumption or incorporation into protein synthesis. Lysine, an essential amino acid, showed strong variability, with marked enrichment in *Lb. paracasei* AF43 (almost 15 %) *Lb. delbrueckii* subsp. *bulgaricus* LbL9 (2 %) and *Ent. malodoratus* A2 (7 %). Interestingly, fermentation also promoted the appearance of histidine in some samples, notably in *Lb. delbrueckii* subsp. *lactis* LbL33, where it reached higher relative levels compared to the unfermented WM, where it was not detected. Similarly, phenylalanine, which was absent in WM, emerged in *Lb. delbrueckii* subsp. *lactis* LbL37. Other amino acids, such as cysteine, alanine, leucine, cystine, and threonine, maintained low relative abundances and showed only minor variations among strains. These observations align with the amino acid requirements of LAB, which generally depend on branched-chain and aromatic amino acids, as well as methionine, while showing variable or strain-dependent requirements for histidine, arginine, glutamine, and proline (Chopin, 1993). Accordingly, the accumulation of lysine, proline, alanine, and histidine in some fermentates likely reflects their non-essential or strain-dependent status for LAB, resulting in limited microbial consumption. Overall, these results demonstrate that LAB fermentation modulates the amino acid composition of whey proteins in a strain-dependent manner, reflecting differences in proteolytic capacity. The presence and relative enrichment of essential amino acids (lysine, threonine, leucine, and histidine) and branched-chain amino acids (notably leucine, consistently detected across fermentates) indicate that LAB fermentation did not deplete these key nutrients. Instead, their preservation and, in some cases, fortification underscore the nutritional relevance of fermented whey and its potential to improve the digestibility of whey protein.

The major constituents of whey proteins are β-Lg, α-La, BSA, and some residual casein-derived oligopeptides, mainly CMP, i.e., the hydrophilic fragment of k-casein released during enzymatic coagulation of caseins (Olvera-Rosales et al., 2023). LAB are capable of metabolising all these protein fractions, producing fermentates enriched in biologically active peptides with potential health-promoting effects. Identifying which fractions are most susceptible to proteolysis is essential, not only because they represent valuable sources of BPs, but also because specific proteins, such as β-Lg and α-La, are major milk allergens whose degradation can enhance the hypoallergenic properties of whey-based products (Pescuma et al., 2012). In this study, BSA was the most

degraded protein across all strains (Table 3.4), with reduction values often exceeding 85 %, suggesting that BSA is particularly susceptible to LAB proteases.

Table 3.4 Extent of protein hydrolysis (PH%; mean ± standard deviation) by various LAB strains on specific whey protein fractions, including bovine serum albumin (BSA), β-lactoglobulin (β-Lg), α-lactalbumin (α-La), and caseinomacropeptide (CMP)

Strain	BSA	β-Lg	α-La	CMP
Lb. paracasei AF43	$58.43^{b} \pm 4.77$	$13.66^{bc} \pm 5.38$	$21.24^{bd} \pm 2.27$	$61.02^{ac} \pm 8.01$
Lb. plantarum 297	$92.49^{a} \pm 1.04$	$2.44^{cd} \pm 0.42$	$11.95^{d} \pm 4.69$	$46.55^{c} \pm 8.29$
St. macedonicus LbL43	$91.8^{a} \pm 4.36$	$0.80^{d} \pm 0.13$	$12.92^{d} \pm 0.29$	$52.29^{ac} \pm 3.42$
Lb. delbrueckii subsp. *delbrueckii* LbL38	$89.04^{a} \pm 4.04$	$27.72^{ab} \pm 2.37$	$32.2^{bc} \pm 4.18$	$59.96^{ac} \pm 3.48$
Lb. delbrueckii subsp. *bulgaricus* LbL9	$85.13^{a} \pm 7.40$	$18.72^{abc} \pm 8.1$	$30.24^{cd} \pm 8.90$	$65.02^{ac} \pm 5.02$
Lb. delbrueckii subsp. *delbrueckii* LbL39	$93.29^{a} \pm 1.51$	$30.31^{ab} \pm 2.57$	$36.13^{bc} \pm 4.97$	$60.31^{ac} \pm 4.03$
Lb. delbrueckii subsp. *lactis* LbL37	$99.12^{a} \pm 0.06$	$28.3^{ab} \pm 5.67$	$39.87^{a} \pm 8.15$	$66.17^{bc} \pm 2.15$
Lb. delbrueckii subsp. *lactis* LbL33	$94.35^{a} \pm 2.28$	$33.2^{a} \pm 1.30$	$36.78^{bc} \pm 4.66$	$60.25^{ac} \pm 4.36$
Ent. malodoratus A2	$95.17^{a} \pm 0.87$	$12.55^{bc} \pm 6.99$	$26.43^{cd} \pm 0.01$	$86.11^{a} \pm 6.06$
Lb. delbrueckii subsp. *delbrueckii* LbL12	$87.06^{a} \pm 1.88$	$28.21^{ab} \pm 5.78$	$30.79^{abc} \pm 2.60$	$64.27^{ac} \pm 6.01$
Lb. delbrueckii subsp. *delbrueckii* LbL14	$85.67^{a} \pm 0.81$	$30.49^{ab} \pm 2.26$	$30.78^{abc} \pm 0.54$	$67.74^{ab} \pm 3.93$
Lb. delbrueckii subsp. *delbrueckii* LbL2	$84.64^{a} \pm 11.52$	$12.04^{bc} \pm 5.19$	$30.64^{abc} \pm 4.04$	$15.42^{c} \pm 3.26$

Statistical difference ($p<0.05$) within each column is indicated by different letters

The most efficient BSA hydrolysis was observed in *Lb. delbrueckii* subsp. *lactis* LbL37, with a reduction value almost reaching 100 %. By contrast, β-Lg and α-La were generally more resistant to proteolysis. The hydrolysis of β-Lg remained below 20 % for most strains, except for LbL33, which reached a maximum reduction of 33.2 ± 1.3 %. Similarly, α-La degradation was generally low, ranging from 11.95 ± 4.69 % (*Lb. plantarum* 297) to 39.87 ± 8.15 % (*Lb. delbrueckii* subsp. *lactis* LbL37). Although LAB proteolytic systems can act on all whey protein fractions, they typically show a preference for caseins (Solieri et al., 2022). Consistently, CMP appeared to be moderately hydrolysed, with most strains achieving reductions between 45 % and 67 %. Notably, *Ent. malodoratus* A2 showed the highest reduction of this fraction (86.11 ± 6.06 %), despite a moderate total proteolytic activity (31.25 ± 1.34 %), suggesting a possible specificity of its proteolytic system toward this fraction. Overall, *Lb. delbrueckii* subsp. *lactis* strains displayed the most extensive and balanced proteolytic activities, efficiently degrading multiple whey protein fractions and potentially contributing both to improved digestibility and reduced allergenicity.

3.3.2 Evaluation of the Biological Properties of the Fermentates

3.3.2.1 Antioxidant Activity

Antioxidant activity is relevant both in the food industry, where various substances are prone to oxidation with a negative impact on food products, and physiologically, as the oxidation of cellular components triggers aging and cellular degeneration (Tonolo et al., 2024; Zhu et al., 2024). Several mechanisms are implied in the antioxidant capacity of food matrices (Marazza et al., 2012). Therefore, the antioxidant activity of the cell-free fermentation supernatants was assessed in terms of DPPH and FRAP (Figure 3.1).

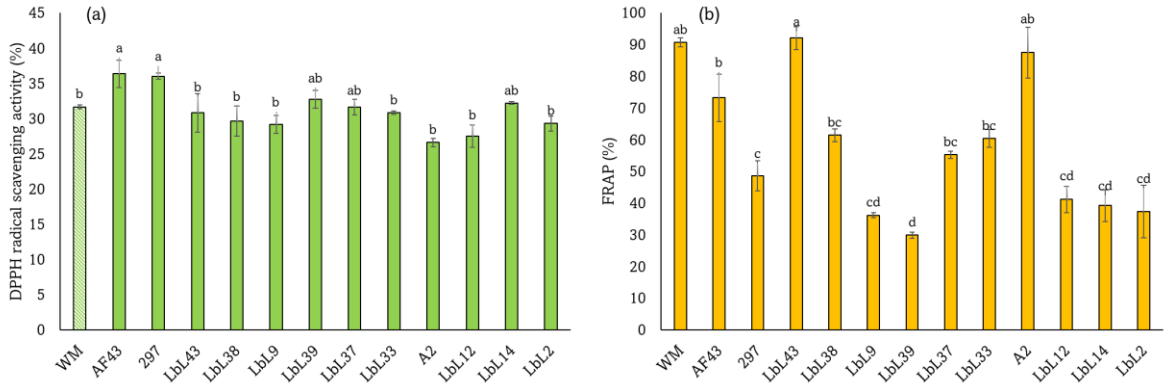

Figure 3.1 Antioxidant activity (mg TE/g protein) determined by DPPH (a) and FRAP (b) assays of whey medium (WM) and fermentates. Data are expressed as mean ± standard deviation. Statistical differences (p<0.05) are indicated by different letters

WM exhibited a certain radical scavenging activity (31.66 ± 0.29 %) and ferric-reducing ability (90.72 ± 1.44 %), highlighting the antioxidant properties of whey proteins. These findings are consistent with those of other authors (Di Filippo et al., 2024; Lin et al., 2012). The antioxidant activity of native whey proteins is primarily attributed to the high concentration of hydrophobic and aromatic amino acids, which can stabilize radicals through proton or electron donation (Halavach et al., 2024). In addition, the addition of reducing sugars to WM may contribute to its high ferric-reducing capacity, as these compounds are known to enhance FRAP values (Liu et al., 2014). It cannot be excluded that other antioxidant molecules naturally present in milk (e.g., vitamins and minerals) and that may remain in the WPC also contribute to the overall antioxidant activity (Khan et al., 2019).

Fermentation modulated the DPPH radical scavenging activity of WM, with values ranging from 26.62 ± 0.61 % to 36.36 ± 1.92 %. The best performances were observed for *Lb. plantarum* 297 (36.03 ± 0.46 %) and *Lb. paracasei* AF43 (36.36 ± 1.92 %), which exceeded the WM by about 15 % (p<0.05). Several other strains displayed radical scavenging capacities comparable to WM. Regarding the FRAP assay, the most vigorous ferric-reducing activity was recorded for *St. macedonicus* LbL43 (92.08 ± 3.64 %), which was the only strain to exceed the mean value of the WM.

Ent. malodoratus A2 also displayed a very high reducing capacity, approaching that of WM, while several *Lb. delbrueckii* subspecies and *Lb. plantarum* 297 exhibited substantially lower activity. The generally lower FRAP values of many fermentates compared to WM may, at least in part, be explained by the consumption of reducing sugars during fermentation. Notably, *St. macedonicus* LbL43 and *Ent. malodoratus* A2 were able to sustain high levels of ferric-reducing activity despite this reduction.

Considering that in the medium used the nitrogen fraction represented the major component of the dry weight, except for lactose (which decreased during fermentation), the increase in antioxidant activity could be associated with the accumulation of low-molecular-weight peptides resulting from proteolytic activity, which are known to contribute to antioxidant activity (Shirkhan et al., 2023). However, *Lb. paracasei* AF43, *Lb. plantarum* 297, *St. macedonicus* LbL43, and *Ent. malodoratus* A2 did not release the highest overall peptide quantities (Table 2.3). In contrast, other strains produced considerably higher amounts of small peptides but did not achieve the same antioxidant capacity. This finding suggests that antioxidant capacity is not merely a function of peptide abundance. Instead, it highlights the importance of peptide sequence and composition, since residues such as tyrosine, tryptophan, methionine, cysteine, histidine, and lysine are particularly effective in stabilizing radicals (Di Filippo et al., 2025). Moreover, the structural features of peptides, including their hydrophobicity and basic nature, are likely to play a decisive role in determining their antioxidant potential (Ren et al., 2008). Thus, these strains may have generated peptide fractions with a more favorable composition for antioxidant activity despite their lower overall release of <3 kDa peptides. In particular, *Ent. malodoratus* A2 showed the highest CMP degradation (Table 3.4), suggesting that extensive CMP hydrolysis may contribute to sustaining antioxidant potential. Based on its amino acid composition, CMP represents a promising precursor for the generation of antioxidant peptides, as it can be hydrolyzed into smaller peptide fractions enriched in residues such as glutamate and aspartate, which enhance metal ion chelation, especially with pro-oxidant ions like Fe^{2+} and Cu^{+} (Karimidastjerd & Gulsunoglu-Konuskan, 2023).

In addition to low-molecular-weight peptides, other antioxidant compounds, such as glutathione, exopolysaccharides, or metal-chelating compounds, might also be generated during fermentation. To better elucidate the mechanisms underlying the observed antioxidant activity, it would be essential to characterize the peptide fractions in detail, which would allow the identification of specific peptide sequences responsible for the bioactivity and clarify whether their structure-activity relationships are consistent with previously reported antioxidant motifs.

3.3.2.2 ACE-inhibitory Activity

The effect of fermentation on the ACE-inhibitory activity of whey proteins was evaluated. ACE is an exopeptidase that catalyzes the conversion of angiotensin I to angiotensin II, a potent

vasoconstrictor. Excessive activity of ACE leads to an increase in the production of angiotensin II, resulting in a rise in blood pressure (Olvera-Rosales et al., 2023). Some BPs can bind the active site of ACE with higher affinity than angiotensin I. By binding ACE, BPs prevent angiotensin I from being hydrolyzed into angiotensin II, modulating hypertension (Tondo et al., 2020). In this study, the ACE-inhibitory activity was expressed as a percentage inhibition of ACE (Table 3.5), calculated by comparing the area of the reaction product (hippuric acid) in the presence of the sample with that of a control reaction without an inhibitor.

Table 3.5 ACE-inhibitory activity (%; mean ± standard deviation) of whey medium (WM) and fermentates

Sample	ACE-inhibitory activity (%)
WM	$81.56^{d} \pm 0.06$
Lb. paracasei AF43	$85.87^{a} \pm 0.09$
Lb. plantarum 297	$78.00^{g} \pm 0.01$
St.macedonicus LbL43	$85.27^{a} \pm 0.01$
Lb. delbrueckii subsp. *delbrueckii* LbL38	$78.06^{g} \pm 0.11$
Lb. delbrueckii subsp. *bulgaricus* LbL9	$75.85^{h} \pm 0.30$
Lb. delbrueckii subsp. *delbrueckii* LbL39	$75.96^{h} \pm 0.35$
Lb. delbrueckii subsp. *lactis* LbL37	$79.21^{f} \pm 0.06$
Lb. delbrueckii subsp. *lactis* LbL33	$82.72^{c} \pm 0.12$
Ent. malodoratus A2	$82.33^{c} \pm 0.24$
Lb. delbrueckii subsp. *delbrueckii* LbL12	$83.91^{b} \pm 0.02$
Lb. delbrueckii subsp. *delbrueckii* LbL14	$73.68^{i} \pm 0.18$
Lb. delbrueckii subsp. *delbrueckii* LbL2	$80.71^{e} \pm 0.35$

Statistical differences ($p<0.05$) are indicated by different letters

WM exhibited an inhibition of 81.56 ± 0.06 %, while fermentation modulated this activity ($p<0.05$), with values ranging from 73.68 ± 0.18 % to 85.87 ± 0.09 %, consistent with previous literature (Ahn et al., 2009). Notably, five strains showed an improvement in ACE-inhibitory activity compared to WM, demonstrating that fermentation of whey proteins represents an effective strategy to enhance this functional property. The highest ACE-inhibitory activities were recorded for *Lb. paracasei* AF43 (85.87 ± 0.09 %) and *St. macedonicus* LbL43 (85.27 ± 0.01 %), followed by *Lb. delbrueckii* subsp. *delbrueckii* LbL12 (83.91 ± 0.02 %). By contrast, *Lb. delbrueckii* subsp. *delbrueckii* LbL14, *Lb. delbrueckii* subsp. *bulgaricus* LbL9, and *Lb. delbrueckii* subsp. *bulgaricus* LbL39 showed the lowest ACE-inhibitory effects. It is well known that low-molecular-weight peptides (<3 kDa) may exhibit ACE-inhibitory activity, as this bioactivity is often associated with short peptide sequences containing hydrophobic residues such as proline, isoleucine, tryptophan, phenylalanine, and tyrosine, conferring strong inhibitory potential at the C-terminal region (Li et al., 2004).

Notably, *Lb. paracasei* AF43 and *St. macedonicus* LbL43, which exhibited the strongest ACE inhibition, were not among the best <3 kDa-peptide releasers, suggesting that their effectiveness would probably derive from the release of peptides with particularly favorable sequences rather than from extensive hydrolysis.

Interestingly, these strains also enhanced antioxidant activity, reinforcing the idea that fermentation can simultaneously improve multiple bioactivities, thus representing an effective biotechnological tool for valorizing whey proteins.

3.3.2.3 Antimicrobial Activity

The ability of LAB strains to release compounds with antimicrobial activity may play a crucial role in treating human infections caused by antibiotic-resistant bacteria, as well as in food safety and preservation (Hernández Figueroa et al., 2024). Antimicrobial activity was tested against six pathogens and spoilage bacteria of interest in the food field: three Gram-positive (*Enterococcus* spp., *L. monocytogenes*, and *Staph. aureus*) and three Gram-negative bacteria (*E. coli*, *Salmonella* spp., and *Ps. fluorescens*). Antimicrobial activity was assessed via a turbidimetric approach, considering that the presence of an antimicrobial substance can impact the growth kinetics of a microorganism by extending the lag phase, decreasing the maximum growth rate, or lowering the maximum cell concentration reached at the stationary phase (Innocente et al., 2023). To simplify comparison among growth curves, data were modeled with the Gompertz equation, which allows for estimating the values of the lag time (λ; h), the maximum specific growth rate (μ_{max}; log OD/h), and the amplitude (A; OD), that is, the maximum OD reached by the growth curve. For each genus, target strains were pooled to consider microbial variability in nature. Considering the bioactivities of whey protein against bacteria (Innocente et al., 2023), the growth of strains in WM was first assessed and compared to BHI as a control medium. Table 3.6 describes the effect of the unfermented WM on the growth parameters of the different microbial targets.

The effect of WM on bacterial growth was strain-dependent, showing both antimicrobial and growth-stimulatory properties compared to the control medium (BHI). For *L. monocytogenes* and *Staph. aureus*, WM significantly reduced the lag phase (λ) (p<0.05), indicating enhanced adaptation and earlier growth onset. In *L. monocytogenes*, WM also led to a significantly higher μ_{max}, suggesting a stimulatory effect on growth rate, although the final cell density (A) remained comparable to BHI. Instead, in *Staph. aureus,* WM reduced the final OD, indicating a limited overall biomass yield and an inhibitory action of WM. Similarly, *Salmonella* spp. grown in the presence of WM showed a significantly higher μ_{max} and more extended lag phase, suggesting delayed but accelerated exponential growth, even though the final OD was lower. In contrast, *Enterococcus* spp., *E. coli*, and *Ps. fluorescens* exhibited a significant reduction in final OD (A) in WM, consistent with a moderate inhibitory effect. Additionally, in *Enterococcus* spp., μ_{max} was significantly reduced, and although the

lag phase was shortened, the overall growth was suppressed. These findings indicate that unfermented WM may promote growth in specific pathogens by shortening the lag phase or enhancing the growth rate, while inhibiting others. This dual effect may be due to factors such as nutrient availability (e.g., lactose) or the presence of intrinsic antimicrobial compounds in whey (Innocente et al., 2023).

Table 3.6 Effect of the unfermented whey medium (WM) on growth parameters (mean ± standard deviation) of *L. monocytogenes*, *Staph. aureus*, *Enterococcus* spp., *E. coli*, *Salm. enterica*, and *Ps. fluorescens*

	λ (h)	μ_{max} (log OD/h)	A (OD)
L. monocytogenes			
BHI	$7.11^{a} \pm 0.11$	$0.31^{b} \pm 0.01$	$1.05^{a} \pm 0.01$
WM	$3.47^{b} \pm 0.01$	$0.45^{a} \pm 0.04$	$1.20^{a} \pm 0.06$
Staph. aureus			
BHI	$5.32^{a} \pm 0.50$	$0.52^{a} \pm 0.16$	$1.50^{a} \pm 0.03$
WM	$3.52^{b} \pm 0.08$	$0.43^{a} \pm 0.02$	$1.15^{b} \pm 0.02$
Enterococcus spp.			
BHI	$4.84^{a} \pm 0.05$	$0.85^{a} \pm 0.05$	$1.43^{a} \pm 0.01$
WM	$3.69^{b} \pm 0.00$	$0.64^{b} \pm 0.01$	$1.21^{b} \pm 0.02$
E. coli			
BHI	$2.54^{a} \pm 0.15$	$0.39^{a} \pm 0.02$	$1.47^{a} \pm 0.01$
WM	$2.62^{a} \pm 0.08$	$0.35^{a} \pm 0.01$	$1.33^{b} \pm 0.01$
Salm. enterica			
BHI	$2.74^{b} \pm 0.17$	$0.24^{b} \pm 0.02$	$1.23^{a} \pm 0.02$
WM	$3.51^{a} \pm 0.02$	$0.44^{a} \pm 0.02$	$1.12^{b} \pm 0.01$
Ps. fluorescens			
BHI	$4.67^{a} \pm 0.02$	$0.29^{a} \pm 0.00$	$1.54^{a} \pm 0.01$
WM	$4.64^{a} \pm 0.03$	$0.29^{a} \pm 0.01$	$1.27^{b} \pm 0.02$

μ_{max} is the maximum specific growth rate; λ is the lag time; Amplitude (A) is the upper asymptote
Statistical differences (p<0.05) from the control (BHI) are indicated by different letters

Fermentation significantly improved the antimicrobial properties of WM. Interestingly, *L. monocytogenes* was the most affected. As an example, Figure 3.2 illustrates the growth curves of *L. monocytogenes* in the presence of the unfermented WM (dotted line) and the fermentate of *Ent. malodoratus* A2 (continuous line). As shown, the growth of *L. monocytogenes* was markedly delayed and reduced in the presence of the fermentate compared to WM.

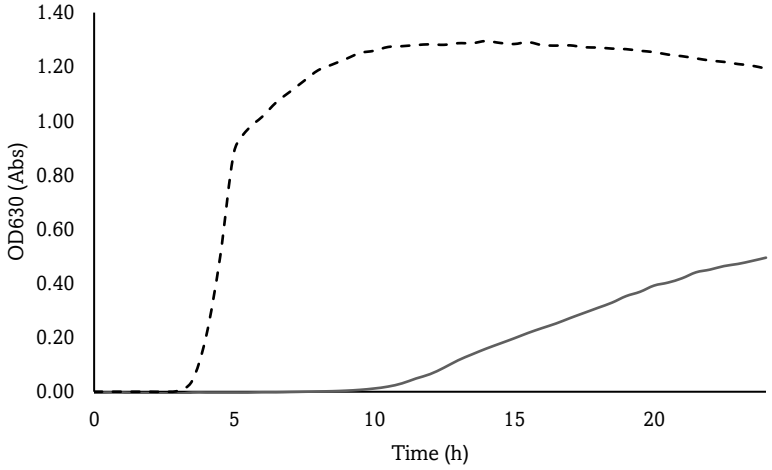

Figure 3.2 Turbidimetric growth curves of *L. monocytogenes* in the presence of the fermentate of *Ent. malodoratus* A2 (continuous line) or the whey medium (WM) (dotted line)

The three-dimensional scatterplots (Figure 3.3) visually summarize the effects of the fermentates and the unfermented WM on bacterial growth, displaying the distribution of the fitted parameters for each microbial target. Although *L. monocytogenes* was stimulated in growth by the presence of WM (Table 3.6), its lag phase was prolonged, and μ_{max} and A decreased (p<0.05) in the presence of all the fermentates, indicating that fermentation of whey protein significantly induced the release of metabolites having antimicrobial properties. The effects of the fermentates on the other target microorganisms varied depending on both the specific target and the fermentate tested. In *Staph. aureus*, the lag phase increased and μ_{max} decreased in the presence of fermentates (p<0.05). However, the final OD was significantly higher than that observed in WM, suggesting that while fermentation enhanced antimicrobial activity, it did not entirely limit growth. For *E. coli* and *Salm. enterica*, the lag phase was shortened (p<0.05), likely due to residual lactose providing initial nourishment, yet both experienced significantly reduced μ_{max} compared to the WM and reached a final OD similar to the BHI control, indicating initial stimulation followed by slowed exponential growth. *Enterococcus* spp. and, to a lesser extent, *Ps. fluorescens* exhibited significantly prolonged lag phases and reduced μ_{max}, suggesting partial inhibition. Interestingly, while WM promoted *Enterococcus* growth relative to BHI, fermentation reversed this trend. *Ps. fluorescens* maintained a higher final OD (p<0.05), implying resilience despite slower kinetics. A notable finding was the strong antimicrobial activity of the fermentate from *Ent. malodoratus* A2, which inhibited all pathogens studied. *Enterococcus* spp., although not considered GRAS nor granted QPS status due to its potential as an opportunistic pathogen and its ability to spread antimicrobial resistance, is nonetheless common in dairy products and, in some cases, valued for its technological contribution. However, its use requires rigorous case-by-case evaluation to ensure safety (Ogier & Serror, 2008). The *Lb. delbrueckii* subsp. *delbrueckii* LbL14 fermentate also exhibited substantial inhibitory effects, particularly against Gram-positive targets.

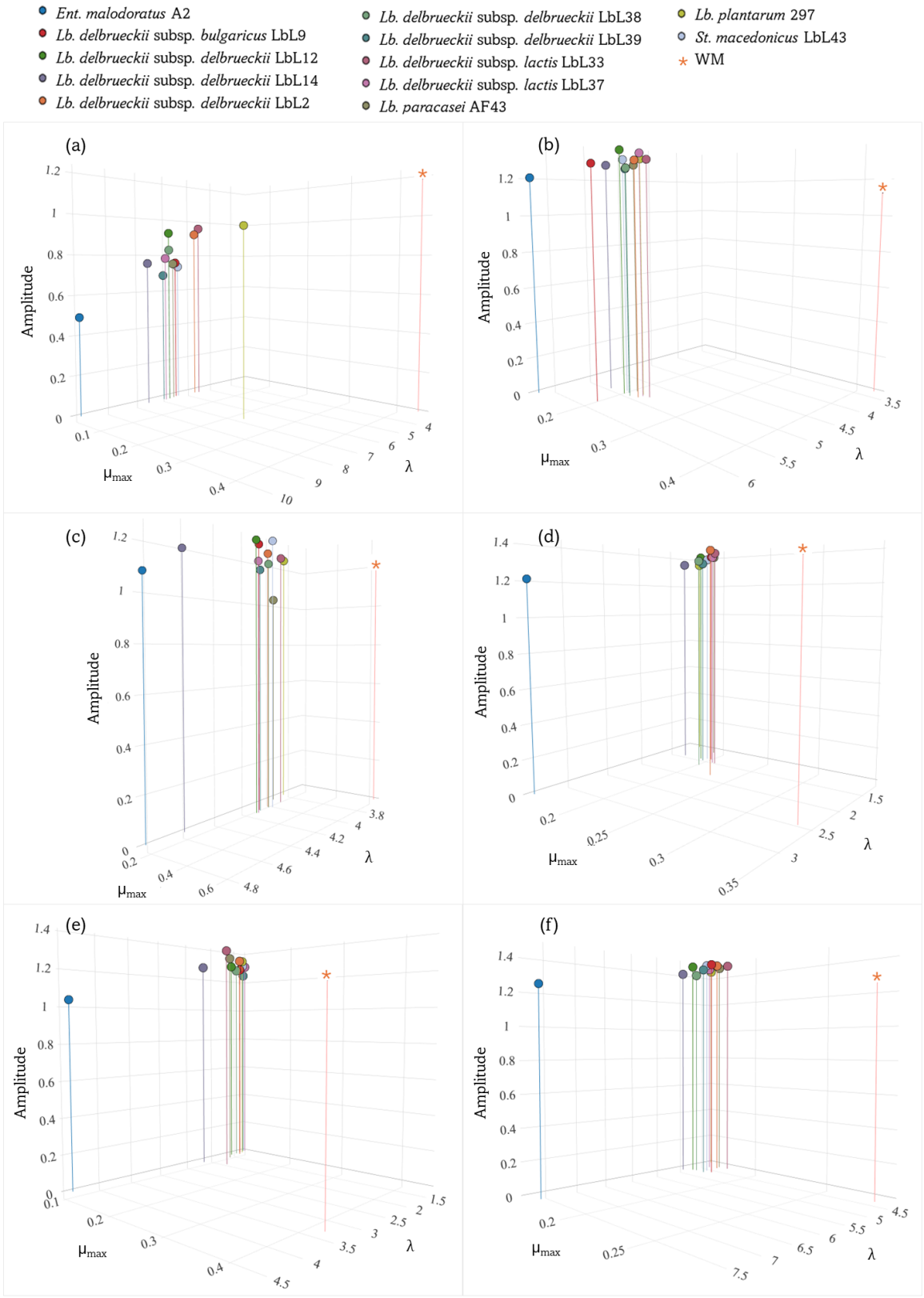

Figure 3.3 Three-dimensional scatterplots showing the effect of the fermentates and the unfermented whey medium (WM) on the growth kinetics of (a) *L. monocytogenes*, (b) *Staph. aureus*, (c) *Enterococcus* spp., (d) *E. coli*, (e) *Salm. enterica*, and (f) *Ps. fluorescens*. The plots represent the fitted growth parameters estimated by the re-parametrized Gompertz model, where Amplitude corresponds to the upper asymptote of the growth curve, μ_{max} is the maximum specific growth rate (log OD/h), and λ represents the lag time (h)

The observed antimicrobial effect, presumably attributable to the release of peptides into the medium, highlights the effectiveness of fermentation. Tested LAB strains produced organic acids during fermentation; however, the pH of the culture medium prepared for the antimicrobial assay after fermentates' addition was approximately 6.6, which excludes an inhibition effect due to acidity. A further potential explanation for the antimicrobial activity seen in this study may be the generation of bacteriocins within the fermentation medium (Guerra et al., 2001). Other metabolites typically associated with LAB, such as exopolysaccharides and hydrogen peroxide, might have also contributed to the observed inhibition (Abdalla et al., 2021; Vieco-Saiz et al., 2019); nonetheless, the increase in peptide prevalence following fermentation strongly supports the hypothesis that peptides are the primary drivers of the antimicrobial activity. The inhibition effect of peptides is derived from their ability to hinder nucleic acids and protein production or by interacting with bacterial membranes (Elsaadany et al., 2024). Peptides bind the bacterial membrane through electrostatic interactions, producing its deformation and an increase in its permeability. The formation of pores across the membrane allows molecules to enter the cell, interfering with the typical metabolic processes of microbes and resulting in reduced cell growth and cell lysis (Olvera-Rosales et al., 2023). Overall, Gram-positive bacteria were more affected than Gram-negative ones. This discrepancy may be due to structural differences in cell envelopes: Gram-negative bacteria possess an outer membrane that may impede BPs from reaching their target sites (Torcato et al., 2013).

3.3.3 Impact on the Gut Microbiota and Its Metabolites

During gastrointestinal transit, most soluble whey-derived metabolites are digested and absorbed in the upper intestine. However, a significant portion of the matrix - including non-absorbed peptides, amino acids, residual sugars, and microbial cells (viable or not) - persists in the undigested fraction that reaches the colon. Notably, it is estimated that about 10 % of the fraction considered absorbable escapes uptake and reaches the colon (Pérez-Burillo et al., 2021). This material becomes available to the resident microbiota, where it can influence both community composition and metabolic activity through cross-feeding interactions, production of SCFA and BCFA, and the release of beneficial components. To mimic this process, we subjected whole fermentates to simulated *in vitro* gastrointestinal digestion and used the resulting undigested fraction for *in vitro* fecal fermentation, allowing us to evaluate its impact on gut microbial ecology and metabolites. To first evaluate the effect of *in vitro* digestion on microbial survival, bacterial counts were assessed before digestion (UND) and after the gastric (G) and intestinal (I) phases. During gastrointestinal transit, microorganisms are exposed to a series of harsh stresses, beginning with low pH and pepsin activity in the stomach, followed by a rapid increase in pH and the presence of bile salts and pancreatic enzymes in the intestine. These conditions can compromise membrane integrity, denature proteins, and reduce metabolic activity, ultimately affecting the number of viable cells able to reach

the colon (Castro-López et al., 2023). As shown in Figure 3.4, all strains exhibited a significant reduction in viability during digestion, although the extent of live cell loss varied in a strain-dependent manner.

For some strains, digestion had a drastic effect, reducing viability to nearly undetectable levels already after the gastric phase (e.g., *St. macedonicus* LbL43, *Lb. delbrueckii* subsp. *delbrueckii* LbL38, LbL37, and LbL33, and *Lb. delbrueckii* subsp. *bulgaricus* LbL9). Among these, *Lb. delbrueckii* subsp. *delbrueckii* LbL38 and *Lb. delbrueckii* subsp. *lactis* LbL37 were no longer detectable at the end of the intestinal phase. Other strains showed a moderate reduction, maintaining final counts above 4 log cfu/mL after intestinal digestion: *Ent. malodoratus* A2, *Lb. delbrueckii* subsp. *delbrueckii* LbL12, LbL2, LbL14, and LbL39. By contrast, *Lb. paracasei* AF43 and *Lb. plantarum* 297 were the most resistant strains, with only minor reductions across both gastric and intestinal phases, maintaining viable counts above 7 log cfu/mL at the end of digestion. Such high survival rates suggest that these strains possess desirable traits for potential probiotic application, as survival through the gastrointestinal tract is considered a key criterion for probiotic selection (Hill et al., 2014). Overall, the gastric phase was the most damaging, accounting for most of the viability loss. In some cases, the intestinal phase further decreased viability, while in resistant strains, the viable count remained stable. These results highlight a wide variability in strain-specific tolerance to digestion, with only a few isolates showing the robustness needed to reach the colon in viable form.

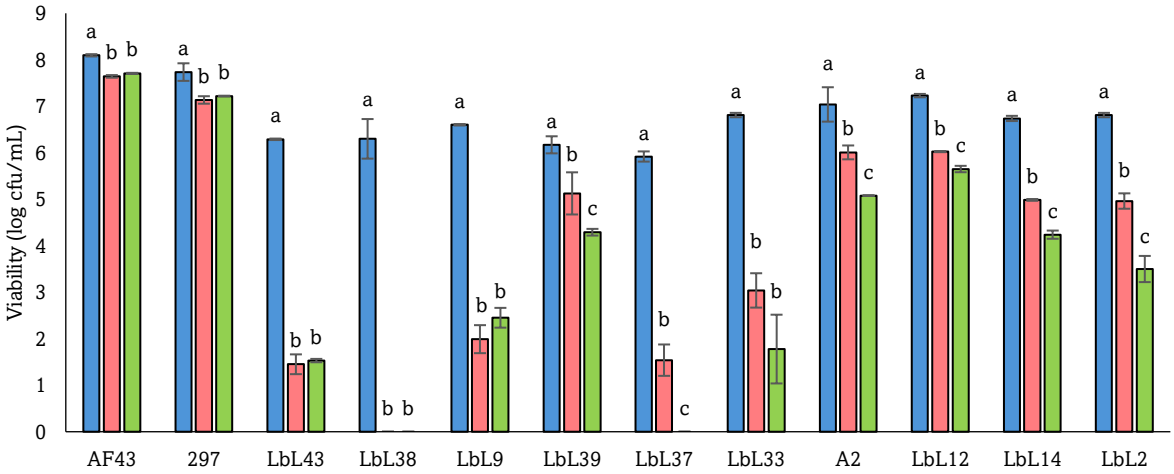

Figure 3.4 Viability (log cfu/mL) of LAB strains before (UND; blue), after gastric (G; red), and after intestinal (I; green) digestion phases. Data are expressed as mean ± standard deviation. Different letters indicate statistically significant differences (p<0.05) among digestion phases within each strain

The undigested fractions obtained after simulated digestion were then subjected to *in vitro* fecal fermentation. In this setup, the pellet fraction collected after fermentation was analyzed by 16S rRNA metagenomic sequencing to assess changes in microbiota composition, while the supernatant was used for the quantification of SCFAs and BCFAs as functional outputs of microbial metabolism. This dual approach allowed us to link community-level shifts with metabolic outputs,

thereby providing an integrated view of the impact of different fermentates on colonic microbial ecology.

Fermentates modulated the fecal microbiota in diverse ways. Microbial profiles resulting from 16S rRNA metagenomic sequencing are reported in Figure S3.1, which displays the relative abundance of the prevalent bacterial species across the different samples. Overall, the analysis proved highly effective, allowing the identification of almost 100 % of the species present and providing a comprehensive overview of the microbial community structure. The barplot shows that the microbial community was mainly dominated by *Bacteroides* spp. This genus contains key commensals involved in polysaccharide degradation and energy harvest (Zafar & Saier, 2021); however, some strains, such as *Bacteroides vulgatus*, have also been associated with pro-inflammatory responses in inflammatory bowel disease patients (Ó Cuív et al., 2017; Zafar & Saier, 2021). The second most abundant species was *Phascolarctobacterium faecium*, a succinate-utilizing bacterium whose growth is supported by cross-feeding with *Bacteroides* spp. and which contributes to the production of propionate, a SCFA with beneficial roles in host metabolism and gut health (Ikeyama et al., 2020). High relative abundance was also found for *Bifidobacterium longum*, a well-recognized commensal and probiotic species involved in carbohydrate fermentation, acetate production, and immune modulation, with documented benefits for host gut health (Fukuda et al., 2012; Vieira et al., 2016). In contrast, the widely detected *Bilophila wadsworthia*, a sulfite-reducing bacterium associated with bile acid metabolism, has been linked to pro-inflammatory processes and has been reported to expand under diets rich in fat and animal protein (Feng et al., 2017).

Alongside these dominant species, several other taxa were detected at lower relative abundance, often in a sample-specific manner. To better understand the biodiversity of the resulting microbial communities, alpha diversity indices were calculated for the WM and the fermentates after *in vitro* colonic fermentation (Table 3.7).

These indices provide complementary information on the structure of the microbial ecosystem: while the number of taxa reflects the number of species identified within each sample, the Simpson and Shannon indices also consider the relative abundance of each species, thus providing insight into community evenness and dominance patterns (Hill et al., 2003). Fermentates displayed variable effects on microbial richness and evenness compared to the unfermented WM. In some cases, no substantial enrichment in diversity was observed (e.g., *Lb. paracasei* AF43, *St. macedonicus* LbL43, and *Lb. plantarum* 297), whereas others showed a clear increase in community complexity. Notably, all samples fermented with *Lb. delbrueckii* strains exhibited higher diversity values (Simpson: 0.86-0.90; Shannon: 2.5-2.8), suggesting the development of a more complex and balanced microbial community after colonic fermentation. Among them, *Lb. delbrueckii* subsp. *bulgaricus* LbL9 displayed the highest indices, indicating the strongest modulation of microbial diversity.

Table 3.7 Alpha diversity indices (Richness, Simpson, and Shannon) of microbial communities detected in whey medium (WM) and in the fermentates after *in vitro* colonic fermentation

Sample	Number of taxa	Simpson index	Shannon index
WM	15	0.81	2.24
Lb. paracasei AF43	15	0.73	1.99
Lb. plantarum 297	15	0.84	2.29
St. macedonicus LbL43	15	0.83	2.32
Lb. delbrueckii subsp. *delbrueckii* LbL38	22	0.90	2.80
Lb. delbrueckii subsp. *bulgaricus* LbL9	25	0.90	2.83
Lb. delbrueckii subsp. *delbrueckii* LbL39	16	0.86	2.48
Lb. delbrueckii subsp. *lactis* LbL37	22	0.90	2.78
Lb. delbrueckii subsp. *lactis* LbL33	19	0.89	2.67
Ent. malodoratus A2	16	0.87	2.52
Lb. delbrueckii subsp. *delbrueckii* LbL12	19	0.89	2.66
Lb. delbrueckii subsp. *delbrueckii* LbL14	23	0.90	2.81
Lb. delbrueckii subsp. *delbrueckii* LbL2	22	0.90	2.76

To explore and visualize differences in microbial community structure across the samples, a PCA was performed (Figure 3.5). PCA revealed that the two principal components (PC1 and PC2) explained 48 % and 17 % of the total variance, respectively, thus capturing the majority of the dataset variability. A clustering analysis was also performed, and the results are shown with a heatmap representation and dendrograms (Figure S3.2). According to the PCA biplot and hierarchical analysis, samples were grouped into four leading clusters (Figure 3.5).

Group I, represented solely by *Lb. paracasei* AF43, appeared as a clear outlier in the lower-left quadrant. Its isolated position indicates a markedly distinct microbial composition. Group II included WM together with fermentates obtained with *Ent. malodoratus* A2, *Lb. plantarum* 297, and *St. macedonicus* LbL43, suggesting that these fermentates maintained a microbial structure largely comparable to the unfermented control. Overall, these two groups were dominated by taxa typically linked to bile-tolerant and proteolytic metabolisms (David et al., 2014; Kaakoush, 2015). In contrast, Group III, comprising several *Lb. delbrueckii* fermentates, was clearly separated from WM and the other samples. This cluster was enriched in saccharolytic and SCFA-producing taxa, which are associated with beneficial gut functions and a eubiotic microbial balance (Bhattacharjee et al., 2025; De Filippis et al., 2022; Liu et al., 2021). Finally, Group IV occupied an intermediate position between Groups II and III, displaying a transitional microbial configuration and suggesting a partial but not complete shift toward a more saccharolytic profile compared to WM.

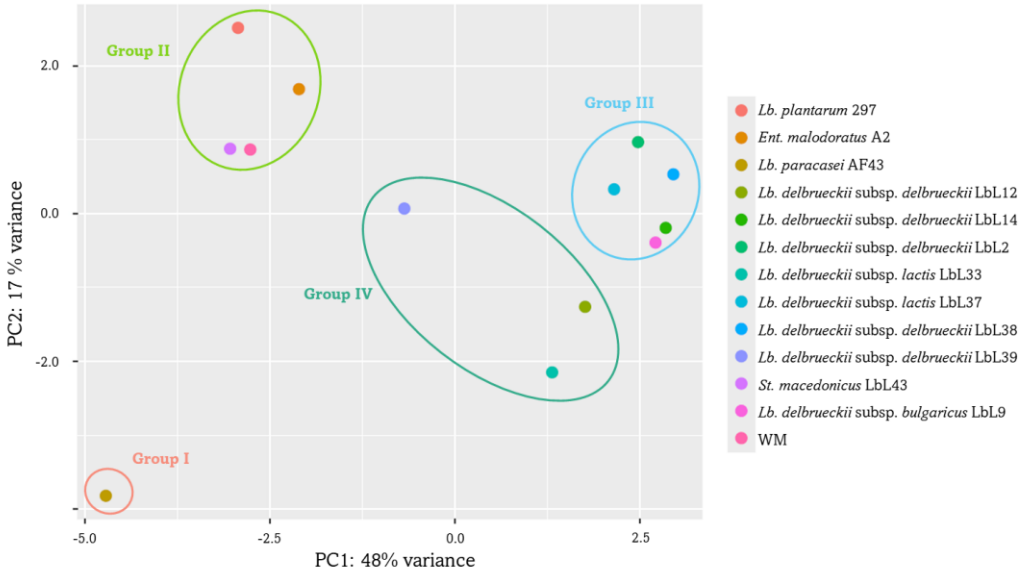

Figure 3.5 Principal component analysis (PCA) biplot for microbial species modulation by *in vitro* colonic fermentation of whey medium (WM) and fermentates. Samples are grouped according to similarity in microbial composition, as identified by hierarchical clustering and heatmap analysis

To better understand the impact of the LAB fermentates on the intestinal microbiota compared to that of the unfermented WM, the $\log_2$ fold change parameter was calculated (Table 3.8). This value was obtained by comparing the relative abundance of each species in the fermentates with that observed in the unfermented WM. Positive values indicate that the abundance of a given taxon increased in the presence of whey fermentates compared to the unfermented WM. To facilitate interpretation of the results, the different taxa were grouped into three functional categories: probiotics and next-generation probiotics, health-supporting commensals, and disease- or dysbiosis-associated microbes. This classification allows us to highlight more clearly the potential impact associated with the presence of LAB fermentates.

Notably, *Akkermansia muciniphila* was significantly enriched in the presence of samples fermented with *Ent. malodoratus* A2, and *Lb. delbrueckii* subsp. *delbrueckii* LbL12, LbL14, and LbL2. *A. muciniphila* is a mucin-degrading bacterium that resides in the gut mucus layer, where it contributes to barrier integrity, immune modulation, and metabolic health. Its abundance has been consistently associated with improved glucose homeostasis, reduced inflammation, and protection against obesity, making it a promising next-generation probiotic (Ioannou et al., 2025). Similarly, *Bifidobacterium longum*, a commensal probiotic involved in carbohydrate fermentation and immune modulation, showed increased abundance in the *Lb. delbrueckii* subsp. *delbrueckii* LbL2 fermentate. As expected, the relative abundance of *Lb. delbrueckii* markedly increased in the presence of fermentates obtained with this microorganism, indicating its ability to persist within the complex microbial community and resulting in a higher representation compared to the unfermented control.

Table 3.8 Log_2 fold change (log_2WM/fermentate) in relative abundance of selected bacterial species after *in vitro* fecal fermentation with different LAB fermentates. Only species with $p<0.05$ are reported. Positive values indicate a relative increase in the fermentate compared to the unfermented whey medium (WM), whereas negative values indicate a relative decrease. Species are grouped according to functional categories: probiotics and next-generation probiotics, health-supporting commensals, and disease- or dysbiosis-associated microbes

Species	AF43	297	LbL43	LbL38	LbL9	LbL39	LbL37	LbL33	A2	LbL12	LbL14	LbL2
Probiotics and Next Generation Probiotics												
Lb. delbrueckii	-38.44	-13.21	-	6.36	7.34	7.32	5.33	7.68	-43.21	7.67	7.4	5.95
Bifidobacterium longum	-	-	-	-	-	-	-	-	-	-	-	0.59
Akkermansia muciniphila	-	-	-	-	-	-	8.8	-	9.47	-	8.71	8.85
Health-supporting commensals												
Blautia obeum	-	-	-	1.12	1	-	0.84	0.68	-	0.64	0.99	1.14
Blautia massiliensis	-	0.84	-	1.62	1.88	-	1.36	-	-0.96	1.34	1.84	1.82
Blautia luti	-6.14	-	-	2.07	2.06	-	-	-	-	-	-	2.05
Blautia faecis	-	-	-	1.4	1.43	-	-	-	-	-	1.26	1.3
Bacteroides uniformis	-	-	-	0.53	-	-	0.58	-	-	-	0.52	0.63
Phascolarctobacterium faecium	-0.45	-	-	-0.83	-0.51	-	-0.4	-	-	-	-0.39	-0.46
Barnesiella intestinihominis	-6.56	-	-7.23	-	-	-	-	-	-	-	-	-
Ruminococcus bromii	-	-8.19	-	-	-	-	-	-	-	-	-	-
Odoribacter splanchnicus	-	-0.86	-	-	-	-	-	-	-	-	-	-
Parabacteroides distasonis	-	-	-	-	-1.01	-	-	-	-	-	-	-
Adlercreutzia equolifaciens	-7.25	-	-	-	-	-	-	-	-	-	-	-
Disease and dysbiosis-associated microbes												
Bilophila wadsworthia	-	-	-	-	-	-	-0.38	-	-	-	-0.37	-
Parasutterella excrementihominis	-	-	-	-0.74	-0.8	-	-	-	-	-	-0.74	-0.69
Turicibacter sanguinis	-	-	-	1.05	-	-	1.05	-	-	-	0.93	1.15
Senegalimassilia anaerobia	-	-	-	1.22	1.31	-	-	-	0.83	-	1.1	1.08

Lb. delbrueckii has been highlighted as a dairy-associated probiotic species with the capacity to release bioactive metabolites and to interact beneficially with the gut ecosystem, supporting both microbial balance and host protection (Latif et al., 2023).

Among health-supporting commensals, several members of the genus *Blautia* were positively modulated by LAB fermentates, showing significant increases in *Lb. delbrueckii* LbL38, LbL9, LbL14, and LbL2 fermentates, which were originally derived from fermentations with *Lb. delbrueckii*. Members of the genus *Blautia* have recently attracted attention as a new functional group with potential probiotic properties, given their involvement in SCFA production, modulation of host immunity, and overall contribution to gut homeostasis (Liu et al., 2021). Also, *Bacteroides uniformis* was increased after fecal fermentation of *Lb. delbrueckii* LbL38, LbL37, LbL14, and LbL2 fermentates. This species has been reported as a health-supporting commensal with anti-inflammatory properties and a role in maintaining intestinal barrier function, further suggesting that certain LAB fermentates may contribute to beneficial microbial modulation (Fabersani et al., 2021). By contrast, some other beneficial commensals, such as *Parabacteroides distasonis, Barnesiella intestinihominis,* and *Adlercreutzia equolifaciens,* were reduced following the addition of some of the fermentates (e.g., samples *Lb. paracasei* AF43 and *Lb. delbrueckii* subsp. *bulgaricus* LbL9). Similarly, *Ruminococcus bromii* and *Odoribacter splanchnicus* were negatively modulated in sample *Lb. plantarum* 297. All these species share common functions such as SCFA production, anti-inflammatory and anti-obesity activity, and support of metabolic homeostasis, making them relevant markers of a healthy gut microbiota (Kim et al., 2024b; Oñate et al., 2023; Wang et al., 2019; Zhang et al., 2025b; Zhuang et al., 2025). Interestingly, *Phascolarctobacterium faecium*, typically sustained by cross-feeding from *Bacteroides* spp. (Ikeyama et al., 2020), remained relatively stable, showing only slight decreases in a few fermentates.

Among taxa associated with dysbiosis, *Bilophila wadsworthia* displayed reductions in the presence of some *Lb. delbrueckii* fermentates (LbL37 and LbL14). This is particularly relevant since it was one of the more prevalent species in the samples in terms of relative abundance (Figure S3.1). Similarly, *Parasutterella excrementihominis*, often linked to irritable bowel syndrome and intestinal chronic inflammation (Galley et al., 2024), decreased in several samples. In contrast, *Turicibacter sanguinis* and *Senegalimassilia anaerobia,* species associated with pro-inflammatory activity, metabolic disorders, and dysbiotic states (Kling et al., 2024), were enriched in various samples, indicating that certain fermentates may promote the growth of undesirable taxa.

The metabolic activity of the fecal microbiota during colonic fermentation was reflected in SCFA and BCFA production, metabolites that contribute to energy harvest, epithelial barrier function, and immune signaling (Wang et al., 2024b). After 24 h of fecal fermentation, clear differences in SCFA and BCFA concentration were observed depending on the strain tested (Table 3.9).

Table 3.9 Concentrations of short-chain fatty acids (SCFA, mM) and branched-chain fatty acids (BCFA, mM) produced during *in vitro* fecal fermentation of the undigested fraction of whey medium (WM) and of protein fermentates obtained from whey protein by fermentation with selected strains. Data are expressed as mean ± standard deviation

	SCFA (mM)				BCFA (mM)	
	Formic acid	Acetic acid	Propionic acid	Butyric acid	Isobutyric acid	Isovaleric acid
WM	$10.04^{b} \pm 0.37$	$45.86^{b} \pm 1.23$	$15.02^{e} \pm 0.26$	$4.03^{c} \pm 0.03$	$0.37^{c} \pm 0.03$	$1.75^{c} \pm 0.01$
Lb. paracasei AF43	$6.43^{d} \pm 0.49$	$32.78^{e} \pm 0.57$	$20.35^{c} \pm 0.04$	n.d.	n.d.	$1.84^{c} \pm 0.91$
Lb. plantarum 297	$8.97^{c} \pm 0.17$	$37.24^{d} \pm 0.11$	$24.56^{b} \pm 0.18$	$2.88^{d} \pm 0.18$	$0.21^{c} \pm 0.01$	$1.71^{c} \pm 0.01$
St. macedonicus LbL43	$10.32^{b} \pm 0.14$	$43.95^{c} \pm 0.11$	$16.24^{e} \pm 0.10$	$4.25^{c} \pm 0.16$	$0.47^{c} \pm 0.3$	$3.04^{b} \pm 0.31$
Lb. delbrueckii subsp. *delbrueckii* LbL38	$12.27^{a} \pm 0.91$	$47.21^{b} \pm 0.71$	$17.06^{e} \pm 0.33$	$4.98^{b} \pm 0.25$	$0.11^{c} \pm 0.08$	$3.27^{a} \pm 0.24$
Lb. delbrueckii subsp. *bulgaricus* LbL9	$14.46^{a} \pm 1.02$	$53.9^{a} \pm 2.25$	$17.61^{d} \pm 0.14$	$7.74^{a} \pm 0.54$	n.d.	$2.03^{b} \pm 0.10$
Lb. delbrueckii subsp. *delbrueckii* LbL39	$11.47^{b} \pm 1.24$	$46.16^{b} \pm 0.63$	$16.37^{e} \pm 0.66$	$4.91^{b} \pm 0.06$	$0.28^{c} \pm 0.06$	$3.03^{b} \pm 0.13$
Lb. delbrueckii subsp. *lactis* LbL37	$10.65^{b} \pm 0.22$	$49.42^{ab} \pm 0.77$	$23.93^{b} \pm 0.72$	$2.94^{d} \pm 0.15$	$1.14^{b} \pm 0.19$	$3.61^{a} \pm 0.16$
Lb. delbrueckii subsp. *lactis* LbL33	$11.13^{b} \pm 0.79$	$46.99^{b} \pm 0.19$	$22.63^{b} \pm 0.72$	$1.12^{e} \pm 0.15$	$0.17^{c} \pm 0.06$	$2.34^{b} \pm 0.11$
Ent. malodoratus A2	$9.77^{b} \pm 0.13$	$38.97^{d} \pm 0.21$	$24.65^{b} \pm 1.53$	$4.95^{b} \pm 0.21$	$0.16^{c} \pm 0.01$	$1.60^{c} \pm 0.01$
Lb. delbrueckii subsp. *delbrueckii* LbL12	$13.39^{a} \pm 0.01$	$51.35^{a} \pm 0.77$	$19.65^{d} \pm 0.15$	$4.08^{c} \pm 0.26$	$2.02^{a} \pm 0.15$	$3.38^{a} \pm 0.28$
Lb. delbrueckii subsp. *delbrueckii* LbL14	$13.42^{a} \pm 0.15$	$47.01^{b} \pm 0.43$	$17.65^{d} \pm 0.22$	$4.02^{c} \pm 0.13$	$0.84^{c} \pm 0.10$	$3.18^{b} \pm 0.14$
Lb. delbrueckii subsp. *delbrueckii* LbL2	$10.92^{b} \pm 0.01$	$46.82^{b} \pm 0.37$	$29.77^{a} \pm 0.71$	$4.68^{c} \pm 0.10$	n.d.	$2.94^{b} \pm 0.18$

Statistical differences ($p<0.05$) are indicated by different letters
n.d. = not detected

The most abundant metabolite detected across all conditions was acetic acid, followed by propionic, formic, and butyric acid. These results are consistent with previous studies showing that whey protein fermentation modulates SCFA profiles, typically leading to a predominance of acetate, followed by propionate and butyrate (Sánchez-Moya et al., 2017). Compared to the unfermented WM (45.8 ± 1.23 mM), several samples significantly increased acetate production, with the highest level observed for *Lb. delbrueckii* subsp. *bulgaricus* LbL9 (53.9 ± 2.25 mM) and *Lb. delbrueckii* subsp. *delbrueckii* LbL12 (51.35 ± 0.77 mM). By contrast, non-*delbrueckii* fermentates, including *Lb. paracasei* AF43, *Lb. plantarum* 297, *St. macedonicus,* and *Ent. malodoratus* A2 negatively contributed to acetate production. Interestingly, almost all fermentates contributed to an increase in propionic acid production (p<0.05) by fecal microbiota, with levels ranging from 15.0 ± 0.26 mM in WM to a maximum of 29.8 ± 0.71 mM with *Lb. delbrueckii* subsp. *delbrueckii* LbL2. Elevated propionate was also observed in other *Lb. delbrueckii*-derived samples, such as LbL33 and LbL37, as well as in *Lb. plantarum* 297 and *Ent. malodoratus* A2.

This finding is consistent with recent reports highlighting that, besides carbohydrates, dietary proteins and peptides are important substrates for propionate generation during gut fermentation, particularly through metabolic pathways involving *Bacteroides* and certain *Firmicutes* (Rackerby et al., 2024). Propionate plays a key role in maintaining intestinal and metabolic health by being involved in lipid metabolism and immune responses, as well as by exerting anti-inflammatory effects on the colonic epithelium (Reichardt et al., 2014). In the unfermented WM, formic acid accounted for 10.0 ± 0.37 mM. Only a restricted number of fermentates led to a significant increase in its production. In particular, *Lb. delbrueckii* subsp. *bulgaricus* LbL9 and other *Lb. delbrueckii* fermentates, such as LbL12, LbL14, and LbL38 showed higher levels compared to WM. While formate is less studied than the major SCFAs, it may act as an intermediate in microbial cross-feeding, indirectly contributing to hydrogen and methane metabolism (Li et al., 2022a).

In the unfermented WM, butyrate was present at 4.03 ± 0.03 mM, and several fermentates promoted its increase (p<0.05). The strongest effect was observed with the fermentate of *Lb. delbrueckii* subsp. *bulgaricus* LbL9, *Lb. delbrueckii* samples LbL37 and LbL33, and *Ent. malodoratus* A2. The selective rise in butyrate with these fermentates is particularly relevant, given its role as the main energy source for colonocytes, its capacity to reinforce epithelial barrier integrity, and its recognized anti-inflammatory properties. The interplay between gut microbiota and butyrate production has been widely documented, and similar butyrogenic shifts have been reported when whey proteins and obtained products were fermented with human or infant fecal inocula (Smith et al., 2020).

BCFAs, primarily isobutyric and isovaleric acid, arise in the large intestine through microbial fermentation of the branched-chain amino acids valine, leucine, and isoleucine. Structurally, they are one carbon shorter than their amino acid precursors. Because they originate from protein

catabolism, BCFAs have traditionally been regarded as markers of proteolytic fermentation and were often associated with adverse outcomes, such as the accumulation of nitrogenous metabolites and indicators of dysbiosis in the colon (Rios-Covian et al., 2020). However, more recent research has highlighted a more positive role. Chi et al. (2024) demonstrated that protein-based foods generating BCFA can support intestinal development and barrier function in animal models, while Wang et al. (2024b) emphasized their potential involvement in metabolic crosstalk between diet, microbiota, and host health. Thus, while they remain indicators of protein fermentation, BCFAs should not be interpreted exclusively as disadvantageous, but rather as metabolites with context-dependent effects that may balance between health-supporting and adverse outcomes. In the unfermented WM, isobutyric acid was detected at 0.37 ± 0.03 mM, while isovaleric acid reached 1.75 ± 0.01 mM. Compared to these baseline levels, only a restricted number of fermentates markedly modulated BCFA production. For isobutyric acid, a significant increase was observed with the fermentates of *Lb. delbrueckii* subsp. *delbrueckii* LbL12 and, to a lesser extent, with *Lb. delbrueckii* subsp. *lactis* LbL37. Most of the other samples either maintained values similar to WM or showed lower concentrations, and in several, isobutyrate was not detectable. For isovaleric acid, a general increase was observed in most *Lb. delbrueckii* fermentates. The highest levels (p<0.05) were found in *Lb. delbrueckii* subsp. *lactis* LbL37, *Lb. delbrueckii* subsp. *delbrueckii* LbL12, and LbL38 fermentates, with other fermentates such as *Lb. delbrueckii* subsp. *delbrueckii* LbL14, LbL39, and *St. thermophilus* LbL43 also produced significantly higher values compared to WM.

The correlation analysis provided additional insights into the ecological meaning of SCFA and BCFA shifts observed after fermentation (Figure 3.6).

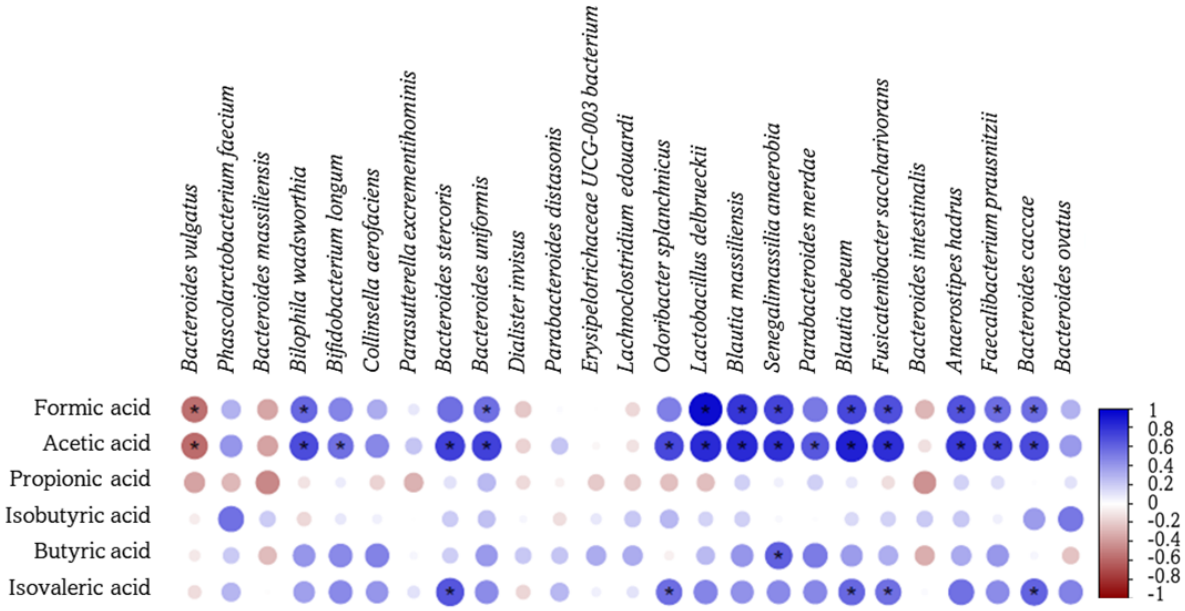

Figure 3.6 Spearman's correlation heatmap between the top 25 microbial taxa for relative abundance (columns) and the concentration of short- and branched-chain fatty acids (SCFA and BCFA) (rows). Positive correlations are shown in blue; negative correlations are shown in red. The circle dimension is proportional to the absolute value of Spearman's correlation coefficient. Significant coefficients (p<0.05) are denoted by an asterisk (*)

An extensive set of significant positive correlations (p<0.05) linked acetate, formate, and isovalerate with well-recognized commensals such as *Bifidobacterium longum*, *Odoribacter splanchnicus*, *Lb. delbrueckii*, several *Blautia* and *Bacteroides* species, *Fusicatenibacter saccharivorans*, *Faecalibacterium prausnitzii*, *Anaerostipes hadrus*, *Bacteroides uniformis,* and *Parabacteroides merdae* (Bhattacharjee et al., 2025; Wang et al., 2024). These taxa are widely described as SCFA and BCFA producers and contributors to gut homeostasis, involved in cross-feeding chains and reinforcing epithelial barrier integrity. Notably, some of these species had already been mentioned for their functional role in SCFA metabolism and beneficial impact on host physiology. Their positive association with acetate and formate supports the idea that whey protein fermentates, particularly those derived from *Lb. delbrueckii*, stimulate beneficial metabolic networks in the gut.

It is also noteworthy that *Bacteroides vulgatus*, a species often associated with dysbiosis and inflammatory conditions, showed significant negative correlations with acetate and formate (p<0.05). This pattern suggests that higher SCFA production may act as a counterbalance against taxa linked to adverse outcomes, reinforcing the health-relevant implications of these metabolic profiles. However, not all associations were clearly beneficial. Some positive correlations (p<0.05) were observed between formate, acetate, or butyrate and species such as *Bilophila wadsworthia* and *Senegalimassilia anaerobia*.

Finally, to explore the relationship between the initial composition of the fermentates and their downstream effects on the gut microbiota, correlation analyses were performed. Since the fermentates obtained from lactic fermentation, used here as a model for protein-based fermented beverages, represent complex mixtures of residual sugars, fermentation metabolites, amino acids, peptides, and unhydrolyzed proteins that can, in part, reach the colon, it is reasonable to assume that these components collectively shape microbial response. Depending on their balance, such mixtures may exert either beneficial or unfavorable impacts on gut community composition and activity. No significant correlations were detected between amino acid and peptide concentration and microbial taxa. However, several significant associations emerged between residual sugars and organic acids of the fermentates and the relative abundance of dominant microbial taxa after fecal fermentation (Figure 3.7).

Galactose, citric acid, and oxalic acid, which were mainly detected in fermentates obtained with *Lb. delbrueckii* strains (Table 3.2), showed positive associations (p<0.05) with *Blautia* spp. and *Anaerostipes hadrus*, as well as with *Lb. delbrueckii* itself. These correlations are consistent with the ability of this species' fermentates to generate products with a strong potential to support beneficial taxa and SCFA production. Significantly, this effect does not stem solely from strain survival through digestion, which was moderate to low for *Lb. delbrueckii* (Figure 3.4), but also from the residual metabolites present in the fermentates. Interestingly, lactic acid was negatively correlated with *Bacteroides vulgatus* (p<0.05), suggesting that residual lactic acid in the fermentates may help

suppress potentially harmful taxa. This is particularly relevant for the fermentate obtained with *Lb. paracasei* AF43, which contained the highest levels of lactic acid.

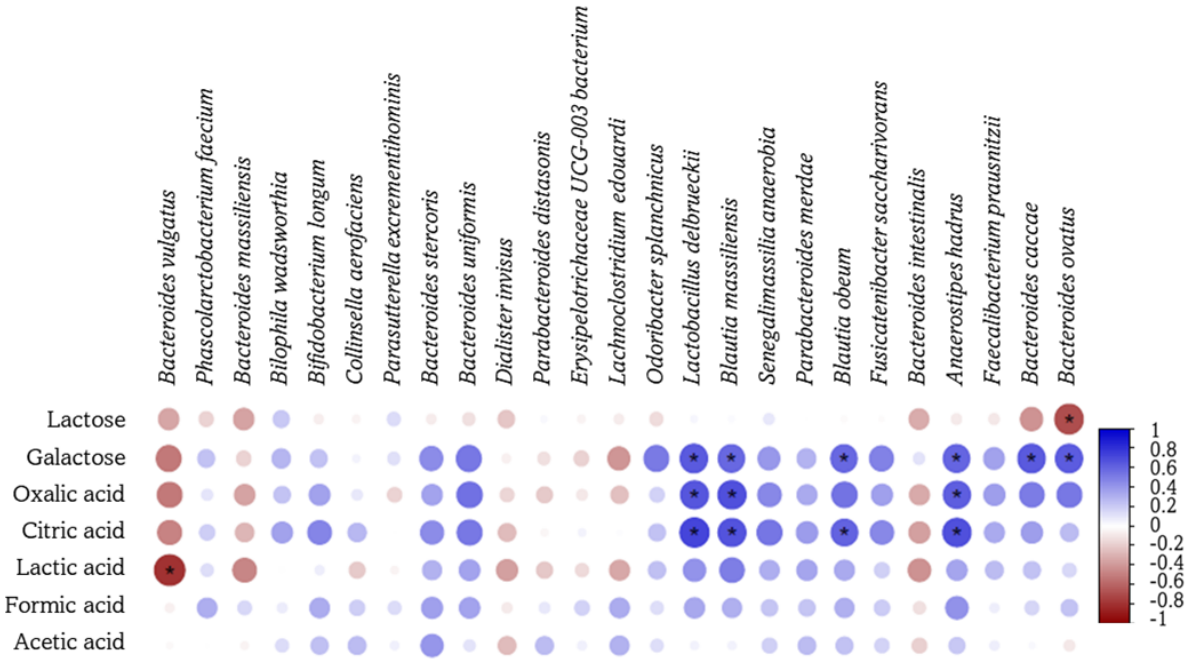

Figure 3.7 Spearman's correlation heatmap between the top 25 microbial taxa for relative abundance (columns) and the concentration of sugars and organic acids detected in the fermentates before *in vitro* digestion and colonic fermentation (rows). Positive correlations are shown in blue; negative correlations are shown in red. The circle dimension is proportional to the absolute value of Spearman's correlation coefficient. Significant coefficients (p<0.05) are denoted by an asterisk (*)

3.4 Conclusions

This study has provided novel insights into the ability of non-starter LAB strains, isolated from raw-milk cheeses and natural milk cultures, to enhance the bioactivities of whey proteins through controlled fermentation. Using the whey medium (WM) as a model for a protein-based fermented beverage, we demonstrated that strain-dependent metabolic activities led not only to the release of distinct peptide fractions but also to the accumulation of other fermentation metabolites, both of which contributed to the functional properties of the final products. The observed strain-specific differences in peptide release and functional enhancement underscore the importance of selecting microbial candidates not only based on proteolytic intensity but also on their capacity to generate targeted low-molecular-weight peptides. Notably, *Lb. paracasei* AF43 stood out by contributing to all three bioactivities: antioxidant, ACE-inhibitory, and antimicrobial, highlighting its potential to drive the development of a multifunctional whey-based fermented beverage with both nutritional and bioprotective value. At the gut level, the complexity of the fermentates, including peptides, amino acids, residual metabolites, and microbial cells, collectively shaped the outcome of fecal fermentation. Several fermentates supported a potentially beneficial modulation of the gut microbiota, promoting health-associated taxa such as *Akkermansia muciniphila* and *Bifidobacterium*

longum. Fermentates derived from *Lb. delbrueckii* strains were especially effective in driving these positive ecological shifts, reinforcing the potential of this group to generate whey-based products with microbiota-targeted benefits. Overall, these findings highlight fermentation as a powerful tool to unlock the bioactive potential of whey proteins and to steer their interaction with the gut microbiota. Future studies should focus on identifying and mechanistically validating the key peptides and other metabolites involved, assessing their stability and functionality in beverage formulations over time, and evaluating their efficacy and safety *in vivo*, thereby supporting the development of functional whey-based fermented beverages.

The results presented in this chapter have been partially published in:

Rossi, A., Innocente, N., Di Filippo, G., Renoldi, N., & Marino, M. (2026). Unlocking the biological potential of whey proteins through lactic acid bacteria fermentation. *Food Bioscience*. DOI: 10.1016/j.fbio.2025.108136.

3.5 Supplementary Material

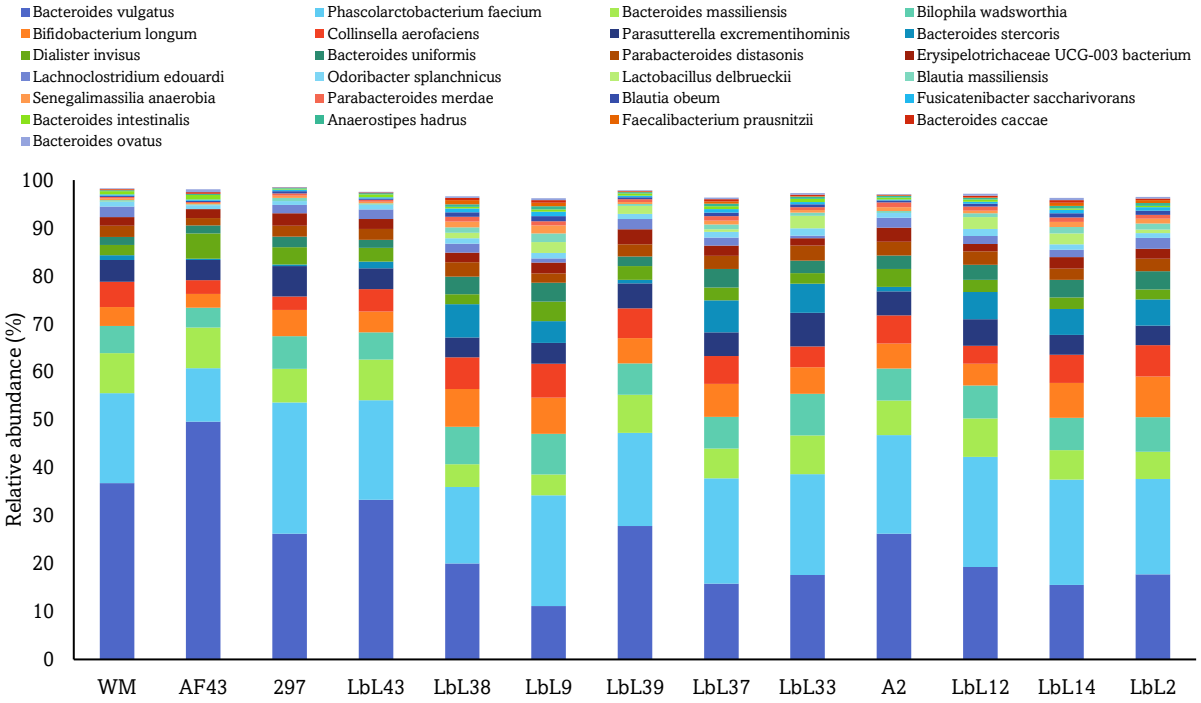

Figure S3.1 Relative abundance (%) of the top 25 microbial species found in the colonic fermented samples of unfermented whey medium (WM) and the fermentates

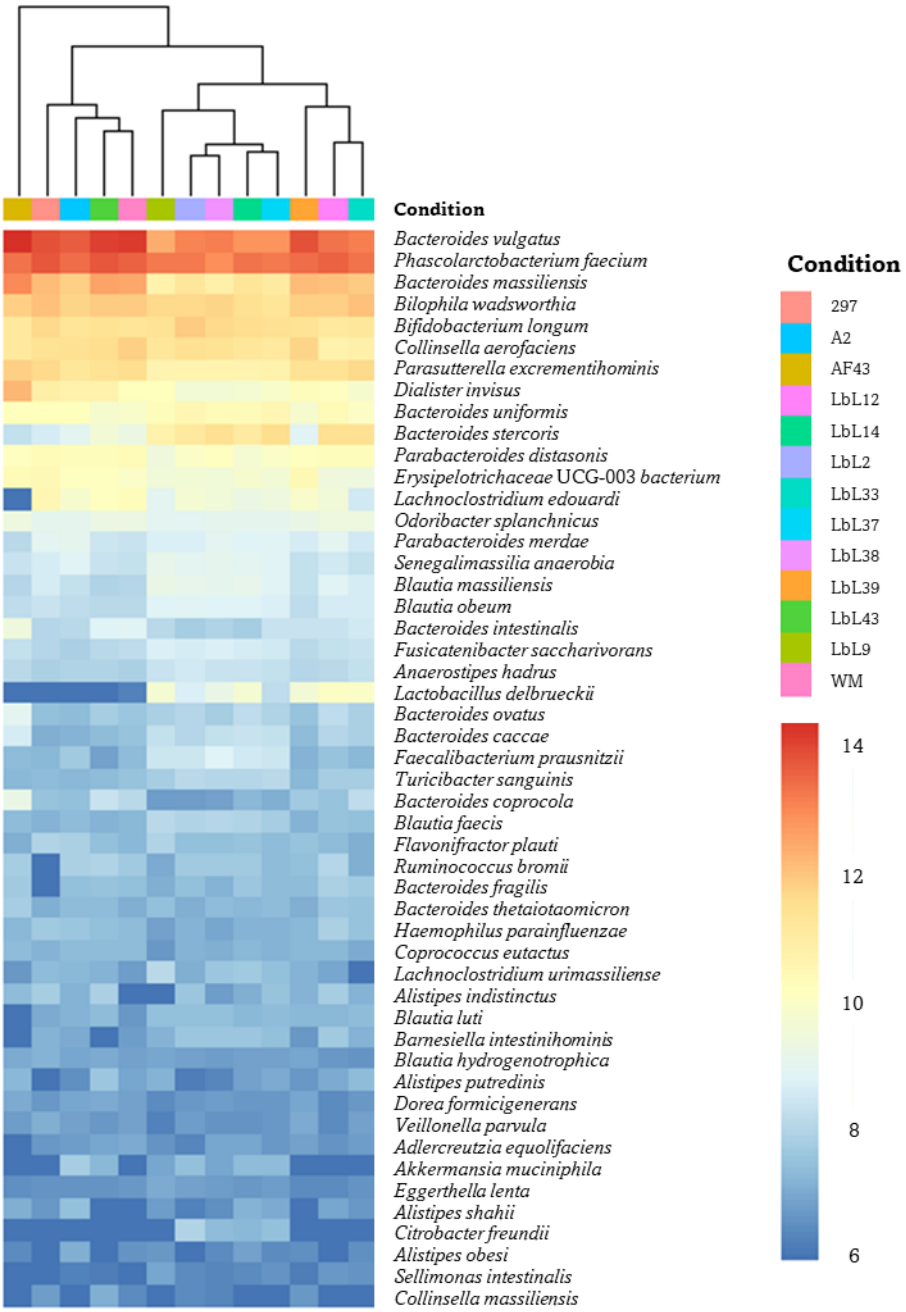

Figure S3.2 Heatmap of microbial community composition after in vitro fecal fermentation of unfermented whey medium (WM) and the fermentates. Samples are clustered according to similarity in taxonomic profiles, with relative abundances of predominant taxa represented by the color scale

Chapter 4:
Boosting the Physiological Benefits of Pea Protein Through LAB Fermentation

4.1 Introduction

Pea (*Pisum sativum* L.) is increasingly recognized as a valuable plant-based protein source, gaining prominence as an alternative to animal-derived proteins, especially in the expanding market for dairy alternatives. Its popularity stems from its relatively high protein content, around 20 % in pea seeds (Farid et al., 2024), as well as its low allergenicity, cost-effectiveness, and favorable nutrient profile. Nevertheless, the use of pea proteins in food applications remains limited by several intrinsic drawbacks, including their lower digestibility and bioavailability compared to animal proteins, mainly due to their compact secondary structure rich in β-sheets (Harper et al., 2022), as well as the presence of undesirable *green* or *beany* off-flavors (Shi et al., 2021).

Fermentation by lactic acid bacteria (LAB) has emerged as a promising strategy to overcome these limitations, improving both the technological and sensory properties of pea proteins (Kravchenko et al., 2024). Several studies have demonstrated that LAB fermentation can effectively reduce undesirable aldehydes, such as n-hexanal, while producing low levels of alcohols and pleasant aroma compounds, thereby improving the sensory quality of pea proteins (Harper et al., 2022; Schindler et al., 2012). At the same time, fermentation has been shown to enhance solubility, emulsion formation and stability, foaming capacity, water-holding ability, and viscosity, essential properties for developing plant-based dairy alternatives with desirable texture and stability (Farid et al., 2024). Beyond these technological improvements, research on the role of fermentation in enhancing the physiological properties of pea proteins remains limited. To the best of our knowledge, only a few studies have addressed this aspect. Babini et al. (2017) and Stanisavljevic et al. (2015) employed different LAB strains to release antioxidant peptides from pea proteins. Similarly, Jakubczyk et al. (2013) used fermentation with *Lactiplantibacillus plantarum,* followed by *in vitro* gastrointestinal digestion, to produce peptides with strong angiotensin-converting enzyme (ACE)-inhibitory activity. Antidiabetic potential has also been reported for fermented legume hydrolysates, which inhibited α-amylase and α-glucosidase (Çabuk et al., 2018). A variety of LAB species have been used for the fermentation of pulses, exploiting their proteolytic systems to hydrolyze proteins and release bioactive peptides (BPs) (Kravchenko et al., 2024; Rizzello et al., 2017). However, the compact, globular conformation of pea proteins (vicilin and legumin) hinders enzymatic accessibility (Harper et al., 2022), highlighting the need for optimized fermentation conditions and careful strain selection tailored to pea-based substrates.

In addition to these bioactivities, increasing attention has been directed toward the impact of plant-derived proteins and their fermentation products on gut microbiota composition and metabolism. Evidence suggests that plant protein hydrolysates, including those derived from peas, can modulate the growth of beneficial bacteria such as lactobacilli and bifidobacteria, thereby enhancing the production of short-chain fatty acids (SCFAs), like acetate, propionate, lactate, and

butyrate, which are crucial for gut health and immune regulation (Swiatecka et al., 2010). Furthermore, a recent clinical trial demonstrated that pea protein supplementation combined with a probiotic significantly altered the gut microbiota profile. It favored beneficial phyla such as *Bacteroidetes*, reducing potentially harmful bacteria, and elevating plasma levels of essential amino acids (Mauricio Retuerto et al., 2023). Nevertheless, studies directly examining pea protein fermentates for gut microbiota modulation remain scarce, underscoring a promising yet underexplored area of research.

In this context, the present study focused on the comprehensive characterization and functional evaluation of fermented pea medium (PM) produced by seven LAB strains as a model of plant-based fermented beverages. Specifically, we assessed sugar consumption, organic acid and amino acid profiles, as well as the functional properties of the fermented products, including antioxidant, ACE-inhibitory, and antimicrobial activities. Furthermore, the impact of these fermentates on gut microbiota composition and microbial metabolite production was evaluated. By integrating metabolic profiling, bioactivity assessment, and gut-related effects, this work aims to provide a comprehensive understanding of the functional potential of LAB-fermented pea protein matrices for the development of sustainable and health-promoting fermented foods (FFs).

4.2 Materials and Methods

4.2.1 Materials

Materials used in this study are listed in Sections 2.2.1 and 3.2.1.

4.2.2 Bacterial Strains and Culture Conditions

The plant-based strains (Table 4.1) isolated and selected in Chapter 2 were stored at -80 °C in MRS broth supplemented with 30 % glycerol (v/v). Cultures were reactivated in 1 mL of appropriate medium, then streaked onto agar plates and incubated for 48 h.

Table 4.1 Bacterial strains used in this study

Species	Strain
Lactiplantibacillus plantarum	CRA
Leuconostoc citreum	C10
Lactiplantibacillus plantarum	M2
Levilactobacillus brevis	C4
Levilactobacillus brevis	C9
Levilactobacillus brevis	C31
Leuconostoc pseudomesenteroides	I3

4.2.3 Characterization of Pea-protein Fermentates

PM was prepared as described in Section 2.2.6.1. Overnight cultures were prepared by inoculating a single colony of each strain in 1 mL of MRS. Each overnight culture was washed once in MRD, inoculated (1 % v/v) in PM, and incubated at 30 °C for 72 h. Uninoculated PM served as a control. At the end of fermentation, supernatant was separated from cells by centrifugation (5,000 × g, 5 min, 4 °C), filtered (0.22 µm), and stored at -20°C for further analysis. Bacterial counts, sugars and organic acids, and free amino acids in the fermentates were determined following the methodologies described in Sections 3.2.3.1, 3.2.3.2, and 3.2.3.3, respectively.

4.2.4 Evaluation of the Biological Properties of the Fermentates

The fermentates and the unfermented PM were assessed for the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (as described in Section 3.2.4.1), the ferric reducing antioxidant power using the ferric reducing antioxidant power (FRAP) assay (Section 3.2.4.2), the ACE-inhibitory activity (Section 3.2.4.3), and the antimicrobial activity (Section 3.2.4.4).

4.2.5 Impact on Gut Microbiota and Its Metabolites

In vitro gastrointestinal digestion was performed according to Section 3.2.5.1, followed by *in vitro* colonic fermentation as described in Section 3.2.5.2. DNA extraction, library preparation, and sequencing were performed according to the procedures outlined in Section 3.2.5.3, and the bioinformatic analysis was performed according to Section 3.2.5.4. The determination of SCFAs and branched-chain fatty acids (BCFAs) was conducted as reported in Section 3.2.5.5.

4.2.6 Statistical Analysis

All trials were carried out at least in duplicate, and values were expressed as the means ± standard deviation. Statistical analysis was performed using R v 4.3.0 (The R Foundation for Statistical Computing, Vienna, Austria). Bartlett's test was used to check the homogeneity of variance. The difference between means was calculated using a t-test and one-way analysis of variance (ANOVA). The Tukey test was used to determine statistically significant differences among means (p<0.05). Principal component analysis (PCA) and hierarchical clustering were performed using the *vegan* package in R (The R Foundation for Statistical Computing). Correlations between the relative abundance of microbial taxa and the concentrations of SCFAs and BCFAs, residual sugars and organic acids, amino acids, and peptides were explored using Spearman's correlation coefficients and visualised with the *corrplot* package in R (https://github.com/taiyun/corrplot). Only taxa and variables with <50 % missing data were included in the correlation analyses.

4.3 Results and Discussion

4.3.1 Characterization of Fermentates Derived from PM Fermentation

Pea proteins are mainly composed of globulins (60–70 %) and albumins (15–20 %), with legumin and vicilin as the predominant storage proteins. These proteins have a compact and closed structure, stabilized by disulfide bonds (in the case of legumin) (Yang et al., 2021). Their structural features not only determine solubility, gelation, emulsification, and foaming, but also play a crucial role in the formulation of plant-based beverages, where stability and texture are key attributes (Boukid et al., 2021). Importantly, although their native structure limits hydrolysis, legumin and vicilin are valuable precursors of BPs that, upon microbial fermentation, can exert health-promoting effects (Aderinola & Duodu, 2022; Kim et al., 2025). These features make pea protein particularly attractive for the development of fermented plant-based beverages, an expanding sector in functional foods. For these reasons, seven proteolytically active LAB strains, isolated from plant-based fermented foods and selected in Chapter 2 for their ability to release low-molecular-weight peptides, were used to ferment PM, a model of a fermented plant-based beverage composed of pea protein concentrate and glucose. All strains efficiently proliferated in the medium, reaching final cell densities of about 7-8 log cfu/mL (data not shown). The composition and functional properties of the resulting fermented beverages were then investigated.

At the end of fermentation, glucose consumption and organic acid production were determined in the samples to uncover strain-specific metabolic patterns and to evaluate their potential impact on the fermented product (Table 4.2). PM contained 54.58 ± 0.13 mg/mL of glucose. LAB generally display different glucose metabolic pathways that strongly influence their end products. Facultative heterofermentative species, such as *Lb. plantarum*, in the presence of excess glucose, primarily converts it into lactic acid via the Embden-Meyerhof-Parnas pathway. However, it can also generate minor amounts of other metabolites depending on the substrate (Wang et al., 2021). In PM, both *Lb. plantarum* strains (CRA and M2) significantly consumed glucose (almost 14 %) and produced the highest lactic acid levels (2.76 ± 0.04 and 3.08 ± 0.05 mg/mL, respectively), consistent with their predominantly homofermentative metabolism. In contrast, obligatory heterofermentative species such as *Leuc. citreum*, *Lb. brevis*, and *Leuc. pseudomesenteroides* metabolize glucose through the phosphoketolase pathway, yielding not only lactic acid, but also acetic acid and, to a lesser extent, other organic acids (e.g., formic acid) (Gänzle, 2015). In this study, heterofermentative strains utilized more glucose from PM than the facultative homofermenters: *Leuc. pseudomesenteroides* I3 exhibited the most significant glucose depletion (23 %), followed by the other obligate heterofermenters (*Lb. brevis* strains and *Leuc. citreum* C10). As for acid production, these heterofermentative strains showed lower lactic acid yields but higher acetic acid yields than *Lb. plantarum* CRA and M2.

Table 4.2 Sugars and organic acids (mg/mL; mean ± standard deviation) detected in pea medium (PM) and fermentates

Strain	Glucose	Lactic acid	Acetic acid	Formic acid	Oxalic acid	Citric acid
PM	$54.58^{a} \pm 0.13$	n.d.	$0.24^{e} \pm 0.01$	$0.16^{b} \pm 0.01$	$0.2^{a} \pm 0.01$	$0.05^{a} \pm 0.01$
Lb. plantarum CRA	$47.27^{b} \pm 0.01$	$2.76^{b} \pm 0.04$	$0.30^{d} \pm 0.02$	$0.18^{ab} \pm 0.03$	n.d.	n.d.
Leuc. citreum C10	$45.34^{c} \pm 0.01$	$1.83^{d} \pm 0.01$	$0.40^{c} \pm 0.01$	$0.19^{a} \pm 0.01$	n.d.	n.d.
Lb. plantarum M2	$46.94^{b} \pm 0.02$	$3.08^{a} \pm 0.05$	$0.32^{d} \pm 0.03$	$0.18^{ab} \pm 0.01$	n.d.	n.d.
Lb. brevis C4	$46.79^{b} \pm 0.25$	$1.79^{e} \pm 0.01$	$0.63^{a} \pm 0.05$	$0.18^{ab} \pm 0.02$	n.d.	n.d.
Lb. brevis C9	$43.37^{d} \pm 0.01$	$2.09^{c} \pm 0.02$	$0.48^{b} \pm 0.01$	$0.16^{b} \pm 0.02$	n.d.	$0.03^{a} \pm 0.01$
Lb. brevis C31	$45.88^{c} \pm 0.07$	$1.74^{e} \pm 0.01$	$0.49^{b} \pm 0.03$	$0.17^{ab} \pm 0.01$	n.d.	$0.05^{a} \pm 0.02$
Leuc. pseudomesenteroides I3	$41.99^{e} \pm 0.01$	$1.15^{f} \pm 0.02$	$0.48^{b} \pm 0.02$	$0.18^{ab} \pm 0.01$	n.d.	$0.06^{a} \pm 0.01$

Statistical difference ($p<0.05$) within each column is indicated by different letters

n.d. not detected

For example, *Lb. brevis* C4 generated 1.79 ± 0.01 mg/mL of lactic acid and 0.63 ± 0.05 mg/mL of acetic acid, whereas *Leuc. pseudomesenteroides* I3, despite showing the highest glucose consumption, produced the least lactic acid, indicating that intermediates were redirected toward acetate rather than lactic acid. *Leuc. citreum* C10 similarly displayed a mixed metabolism, with moderate lactic acid production and appreciable acetic acid, consistent with the canonical heterofermentative profile. Oxalic and citric acids were detectable at low concentrations in the unfermented PM. They disappeared after fermentation in most samples, suggesting that LAB metabolized them during growth. Some strains have been reported to reduce both citrate and oxalate levels in plant-based substrates through enzymatic activity (Eicher et al., 2024; Turroni et al., 2010). In contrast, formic acid was detected in both the unfermented PM (0.16 ± 0.01 mg/mL) and all fermented samples, with little accumulation after fermentation with *Leuc. citreum* C10 (0.19 ± 0.01 mg/mL). This observation suggests that formate may derive from the substrate but is also a by-product of heterofermentative metabolism (Wang et al., 2021). Overall, fermentation of the PM modified its biochemical profile in ways relevant to product quality. *Lb. plantarum* strains favored lactic acid accumulation, resulting in more substantial acidification, whereas heterofermentative strains produced mixed acids, enhancing flavor complexity. Such organic acids have been shown to reduce undesirable beany notes and improve the sensory properties of pea protein matrices (Kaleda et al., 2025). Thus, these results highlight how strain selection can be used to tailor the sensory attributes of plant-based FFs.

Pea protein has a relatively balanced amino acid profile compared to other plant proteins. It generally provides high levels of essential amino acids, such as lysine, leucine, isoleucine, and phenylalanine, but is limited in sulfur-containing amino acids and tryptophan (Gorissen et al., 2018). Analyzing the amino acid profile of fermented pea protein is particularly important, as microbial activity can hydrolyze proteins, thereby modifying the pool and availability of free amino acids and ultimately influencing the nutritional and functional properties of the FF. Table 4.3 shows the relative abundance (%) of free amino acids detected in PM and the fermentates. In PM, the predominant amino acids were leucine and histidine, while the others were present at lower concentrations. This trend was also evident in the fermented samples, although fermentation had a clear strain-dependent impact on the amino acid profile. Leucine showed a significant increase in *Lb. plantarum* CRA (14 %), indicating the ability of this strain to liberate branched-chain amino acids from pea proteins.

In contrast, *Leuc. citreum* C10 and *Lb. brevis* C9 showed a substantial reduction in free leucine relative to PM (7 % and 10 %, respectively), suggesting that these strains may metabolize leucine more actively or redirect it into other metabolic pathways. Histidine also displayed strain-specific behavior: it was slightly enriched in *Leuc. citreum* C10 (almost 10 % increase), indicating efficient release from the protein matrix, whereas *Lb. plantarum* CRA led to a decrease (15 % decrease).

Table 4.3 Relative abundance (%; mean ± standard deviation) of amino acids detected in pea medium (PM) and fermentates

Strain	Histidine	Threonine	Cystine	Alanine	Tyrosine	Methionine	Valine	Leucine
PM	33.48^{ab} ± 6.59	13.88^{a} ± 2.37	10.61^{a} ± 1.89	0.63^{a} ± 0.26	1.07^{a} ± 0.20	4.09^{a} ± 0.84	0.42^{ab} ± 0.10	35.81^{b} ± 1.52
Lb. plantarum CRA	18.01^{b} ± 4.09	14.77^{a} ± 2.70	12.33^{a} ± 4.72	1.08^{a} ± 0.45	1.03^{a} ± 0.23	3.12^{a} ± 0.91	0.26^{b} ± 0.06	49.39^{a} ± 3.02
Leuc. citreum C10	43.24^{a} ± 7.75	10.01^{a} ± 1.41	10.36^{a} ± 2.89	0.82^{a} ± 0.29	0.67^{b} ± 0.11	5.05^{a} ± 1.00	0.61^{a} ± 0.09	29.24^{c} ± 2.15
Lb. plantarum M2	29.17^{ab} ± 7.45	11.88^{a} ± 2.83	12.43^{a} ± 2.93	0.73^{a} ± 0.32	0.16^{c} ± 0.04	4.85^{a} ± 1.25	0.59^{a} ± 0.02	40.20^{ab} ± 8.98
Lb. brevis C4	38.29^{ab} ± 9.29	11.71^{a} ± 2.68	10.86^{a} ± 2.78	0.76^{a} ± 0.40	n.d.	5.29^{a} ± 1.40	0.64^{ab} ± 0.26	32.53^{b} ± 2.97
Lb. brevis C9	34.91^{ab} ± 5.63	16.69^{a} ± 1.56	16.23^{a} ± 2.50	0.77^{a} ± 0.40	n.d.	5.30^{a} ± 0.89	0.40^{ab} ± 0.14	25.72^{c} ± 1.73
Lb. brevis C31	36.11^{ab} ± 7.35	16.66^{a} ± 3.54	11.29^{a} ± 1.73	0.57^{a} ± 0.29	n.d.	5.52^{a} ± 0.69	0.27^{b} ± 0.05	2.56^{b} ± 1.19
Leuc. pseudomesenteroides I3	38.09^{ab} ± 9.57	11.41^{a} ± 2.01	10.15^{a} ± 3.63	0.73^{a} ± 0.38	0.48^{b} ± 0.17	4.95^{a} ± 1.28	0.45^{ab} ± 0.13	33.74^{b} ± 4.28

Statistical differences (p<0.05) within each column are indicated by different letters
n.d. not detected

An increase in free leucine is of particular interest because of its role in stimulating muscle protein synthesis and maintaining metabolic health (Rehman et al., 2023). Histidine can also serve as a precursor for bioactive compounds, such as histamine and histidine-containing dipeptides, which have various physiological functions, including antioxidant, buffering, and immune roles (Brosnan & Brosnan, 2020). Tyrosine was consistently reduced in several fermentates (e.g., it was depleted in samples fermented by *Lb. brevis* strains) compared to the control. This trend suggests that aromatic amino acids serve as preferred substrates for microbial catabolism, potentially contributing to the formation of flavor-active compounds, such as aldehydes and alcohols. Certainly, beyond their nutritional implications, free amino acids can influence the sensory profile of fermented pea-based foods by serving as flavor precursors, thereby improving palatability and consumer acceptance (Flores et al., 2024). For other amino acids such as cystine, threonine, alanine, methionine, and valine, no significant differences were observed, indicating that fermentation did not substantially affect their release or utilization.

4.3.2 Evaluation of the Biological Properties of the Fermentates

4.3.2.1 Antioxidant activity

The antioxidant potential of PM and the fermented samples was determined through two complementary spectrophotometric assays, DPPH and FRAP, which are widely recognized for their robustness, reproducibility, and effectiveness in screening diverse sample sets. Both methods measure absorbance changes following electron-transfer reactions, reflecting different mechanisms of antioxidant action. Specifically, the DPPH assay measures a compound's ability to neutralize stable free radicals by donating a hydrogen atom or an electron. In contrast, the FRAP assay evaluates the reducing capacity of antioxidants by quantifying their ability to convert ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions (Benzie & Strain, 1996; Marazza et al., 2012). Taken together, these methods provide complementary insights into the radical scavenging and electron-donating properties of fermented pea protein. The unfermented PM exhibited moderate intrinsic antioxidant activity (Figure 4.1), with 5.77 ± 0.06 % DPPH radical scavenging activity and 18.51 ± 1.42 % FRAP, which can be attributed to the presence of native bioactive components such as amino acids, small peptides, and residual phenolic compounds naturally occurring in the protein matrix (Pownall et al., 2010; Stanisavljevic et al., 2015). Fermentation significantly influenced the antioxidant profile. However, the extent of the effect was strongly strain dependent. All fermented samples exhibited significantly higher DPPH radical-scavenging activity than PM. *Lb. brevis* C9 showed the highest activity, while *Lb. plantarum* CRA and *Lb. plantarum* M2 had the lowest (p<0.05), although it still showed improvement relative to PM. The increase in DPPH scavenging capacity could be attributed to proteolytic degradation of pea proteins. A comparison of antioxidant activity with the extent of protein hydrolysis and peptide release (Table 2.5) revealed some interesting strain-

dependent trends. In general, strains with a higher degree of proteolysis, such as *Lb. brevis* C9, C4, and C31 and *Leuc. pseudomesenteroides* I3 also showed elevated DPPH radical scavenging activity. This observation suggests that higher proteolysis may favor the release of bioactive sequences with hydrogen- or electron-donating properties. In particular, an increase in the proportion of low-molecular-weight peptides (<3 kDa) was observed in strains with the highest DPPH values, confirming that small peptides contribute to the radical-scavenging capacity, as reported in the literature (Shirkhan et al., 2023).

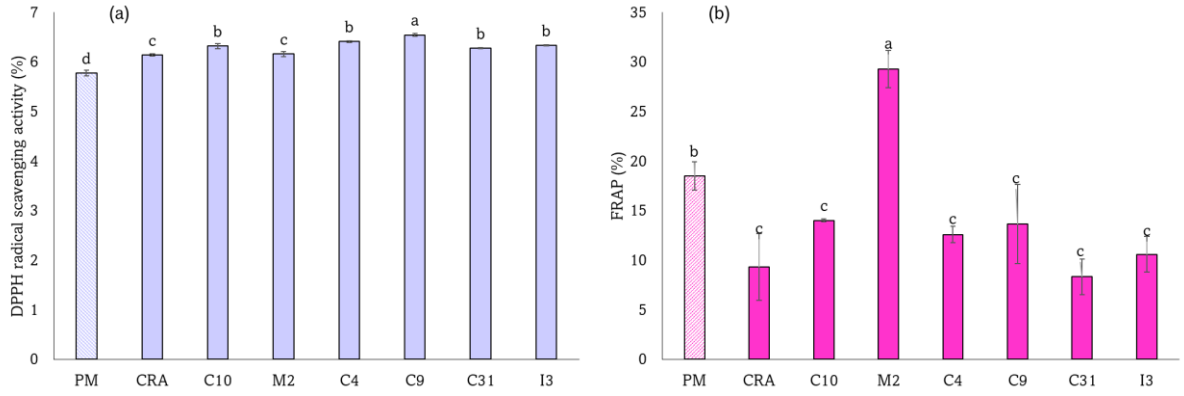

Figure 4.1 Antioxidant activity (%; mean ± standard deviation) determined by DPPH (a) and FRAP (b) assays of pea medium (PM) and fermented samples. Statistical differences (p<0.05) are indicated by different letters

These results are supported by previous studies reporting that batch fermentation of pea proteins with LAB generated products enriched with BPs, which markedly enhanced radical scavenging activity compared to unfermented controls (Stanisavljevic et al., 2015). Similarly, in quinoa flour, fermentation with selected autochthonous LAB strains was shown to improve antioxidant capacity in a strain-dependent manner, further supporting the role of microbial proteolysis and metabolic activity in shaping the antioxidant potential of plant-based protein substrates (Rizzello et al., 2017). Furthermore, microbial activities during fermentation may release phenolic derivatives or transform bound phenolics into more bioavailable forms, enhancing antioxidant potential (Çabuk et al., 2018). Certain LAB strains can also produce exopolysaccharides with free hydroxyl groups capable of scavenging radicals and chelating metal ions (Bisson et al., 2024).

In contrast, the FRAP assay didn't reveal this trend, showing greater differences among the strains (Figure 4.1). While most fermentations significantly decreased FRAP compared to PM, *Lb. plantarum* M2 markedly improved it (p<0.05), reaching values almost double those of the control (29.28 ± 1.88 %), despite a moderate degree of hydrolysis and only average peptide release (Table 2.5). This hydrolysate appeared particularly effective, suggesting that the quality and composition of the released peptides, rather than their overall abundance, may play a role in antioxidant capacity. Additionally, the observed increase in antioxidant activity might also be due in part to the release or transformation of non-peptidic antioxidants during fermentation, such as phenolic compounds or glutathione (Dissanayake et al., 2025). Further targeted metabolomic and peptide sequencing

analyses would be necessary to identify the specific compounds responsible for the bioactivity and to clarify the relative contribution of each metabolic pathway.

4.3.2.2 ACE-inhibitory activity

The effect of fermentation on the ACE inhibitory activity of PM was also tested. ACE plays a central role in blood pressure regulation by generating angiotensin II, a potent vasoconstrictor. Excessive ACE activity is therefore linked to the development of hypertension. Pharmacological ACE inhibitors are widely used in therapy, but their administration may lead to adverse effects. This has stimulated interest in food-derived BPs as natural ACE inhibitors with fewer side effects (Olvera-Rosales et al., 2023; Tondo et al., 2020). The standard *in vitro* method uses the substrate hippuryl-histidyl-leucine. When ACE is active, it hydrolyzes the substrate, releasing hippuric acid, which HPLC can detect. In the presence of an inhibitor, the enzymatic activity is reduced, resulting in a smaller peak area of the reaction product. The percentage decrease in the hippuric acid peak area, therefore, reflects the ACE-inhibitory potential of the sample and its possible antihypertensive relevance (Cushman & Cheung, 1971). In this study, the unfermented PM exhibited the highest activity (81.84 ± 0.07 %) (Table 4.4).

Table 4.4 ACE-inhibitory activity (%; mean ± standard deviation) of pea medium (PM) and fermented samples. Statistical differences (p<0.05) are indicated by different letters

Strain	ACE inhibitory activity (%)
PM	81.84^{a} ± 0.07
Lb. plantarum CRA	77.80^{d} ± 0.03
Leuc. citreum C10	78.90^{c} ± 0.17
Lb. plantarum M2	71.00^{f} ± 0.11
Lb. brevis C4	80.28^{b} ± 0.19
Lb. brevis C9	74.47^{e} ± 0.22
Lb. brevis C31	67.58^{g} ± 0.20
Leuc. pseudomesenteroides I3	80.36^{b} ± 0.12

Statistical difference (p<0.05) among fermentates is indicated by different letters

Overall, fermentation with LAB did not improve ACE inhibitory activity, indicating that the tested strains' proteolytic systems were unable to generate peptides with greater inhibitory activity. In contrast to the present findings, the literature indicates that pea protein hydrolysates, as well as hydrolysates from other plant sources, generally exhibit enhanced ACE-inhibitory activity. This effect has been observed not only after fermentation but also following enzymatic hydrolysis (Chourasia et al., 2024; Di Filippo et al., 2025). An interesting approach is to combine the two strategies. Indeed, Jakubczyk et al. (2013) demonstrated that while fermentation of pea protein alone did not significantly improve ACE inhibitory properties, the sequential application of

fermentation followed by simulated gastrointestinal digestion resulted in a higher release of active peptides. These observations suggest that although LAB alone may not efficiently generate intense ACE-inhibitory sequences, fermentation can serve as a proper pretreatment that facilitates the subsequent enzymatic release of BPs.

4.3.2.3 Antimicrobial activity

Pea protein hydrolysates are increasingly recognized for their antimicrobial potential (Mohan et al., 2021; Wang et al., 2025). LAB can release a wide range of BPs during fermentation, which are of interest for both managing antibiotic-resistant pathogens and improving food safety and preservation (Hernández Figueroa et al., 2024). To investigate this activity, six food-relevant pathogens and spoilage organisms were selected as targets: three Gram-positive (*Enterococcus* spp., *Listeria monocytogenes*, *Staphylococcus aureus*) and three Gram-negative bacteria (*Escherichia coli*, *Salmonella enterica*, *Pseudomonas fluorescens*). In this study, antimicrobial activity was assessed using a turbidimetric assay, which monitors changes in growth dynamics indicative of inhibition (Innocente et al., 2023). Growth data were modeled using the Gompertz equation to extract standardized kinetic parameters: lag time (λ, h), maximum growth rate (μ_{max}, log OD/h), and growth amplitude (A, OD), which represent the upper asymptote. Pooled cultures were employed to reflect natural strain variability. Preliminary experiments first compared the growth of target microorganisms in the presence of unfermented PM with that in the standard rich medium BHI, used as a control. Results are reported in Table 4.5. Overall, the presence of PM did not affect the growth of Gram-positive targets, as *L. monocytogenes* and *Staph. aureus*, and *Enterococcus* spp. showed growth dynamics comparable to those in BHI. By contrast, Gram-negative bacteria were more markedly influenced. In the presence of PM, *E. coli*, *Salm. enterica*, and *Ps. fluorescens* exhibited altered growth dynamics characterized by faster growth rates but reduced final biomass.

Table 4.5 Effect of the unfermented pea medium (PM) on growth parameters (mean ± standard deviation) of *L. monocytogenes*, *Staph. aureus*, *Enterococcus* spp., *E. coli*, *Salm. enterica*, and *Ps. fluorescens*

	λ (h)	μ_{max} (log OD/h)	A (OD)
L. monocytogenes			
BHI	$7.11^{a} \pm 0.11$	$0.31^{a} \pm 0.01$	$1.05^{a} \pm 0.01$
PM	$7.00^{a} \pm 0.04$	$0.32^{a} \pm 0.01$	$1.16^{a} \pm 0.05$
Staph. aureus			
BHI	$5.32^{a} \pm 0.5$	$0.52^{a} \pm 0.16$	$1.50^{a} \pm 0.03$
PM	$4.90^{a} \pm 0.17$	$0.38^{a} \pm 0.05$	$1.29^{a} \pm 0.07$
Enterococcus spp.			
BHI	$4.84^{a} \pm 0.05$	$0.85^{a} \pm 0.05$	$1.43^{a} \pm 0.01$
PM	$5.04^{a} \pm 0.05$	$0.83^{a} \pm 0.06$	$1.49^{a} \pm 0.05$
E. coli			
BHI	$5.12^{a} \pm 0.15$	$0.41^{a} \pm 0.02$	$1.45^{a} \pm 0.01$
PM	$2.8^{b} \pm 0.01$	$0.45^{a} \pm 0.01$	$1.38^{b} \pm 0.02$
Salm. enterica			
BHI	$2.74^{b} \pm 0.17$	$0.24^{b} \pm 0.02$	$1.23^{a} \pm 0.02$
PM	$4.12^{a} \pm 0.01$	$0.75^{a} \pm 0.05$	$1.01^{b} \pm 0.01$
Ps. fluorescens			
BHI	$4.38^{b} \pm 0$	$0.26^{b} \pm 0$	$1.63^{a} \pm 0$
PM	$5.15^{a} \pm 0.22$	$0.41^{a} \pm 0.04$	$1.37^{b} \pm 0.01$

μ_{max} is the maximum specific growth rate; λ is the lag time; A is the amplitude
Statistical differences ($p<0.05$) from the control (BHI) are indicated by different letters

Fermentation clearly influenced the antimicrobial potential of PM. However, the extent of this effect depended on both the microbial target and the fermenting LAB strain. The most potent inhibitory effects were observed against *L. monocytogenes*. In line with this, an example of the growth dynamics is shown in Figure 4.2, where turbidimetric growth curves of *L. monocytogenes* in the presence of fermentate *Lb. plantarum* M2 are compared with growth in unfermented PM. The figure highlights how fermentation can delay adaptation, reduce μ_{max}, and lower the final biomass.

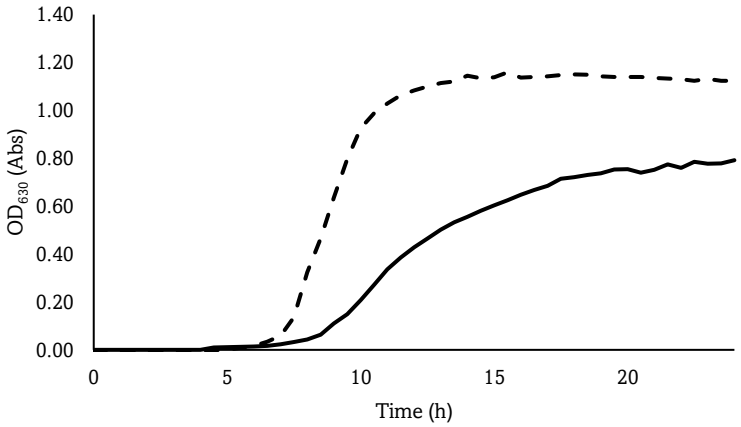

Figure 4.2 Turbidimetric growth curves of *L. monocytogenes* in the presence of fermentate *Lb. plantarum* M2 (continuous line) or the pea medium (PM) (dotted line)

The results of the kinetic parameters for Gram-positive targets in the presence of the fermentates are described in Figure 4.3. As for *L. monocytogenes*, most fermentates significantly prolonged the lag phase, reduced the maximum growth rate, and limited the final biomass in the stationary phase. By contrast, *Staph. Staphylococcus aureus* was only marginally affected by all the fermentates, unlike previous reports showing that pea-derived peptides or protein hydrolysates inhibited this pathogen (Mohan et al., 2021). For *Enterococcus* spp., moderate effects were observed: although some fermentates shortened the lag phase, they subsequently reduced the maximum growth rate and the final biomass accumulation (p<0.05). Among the tested strains, fermentation by *Lb. plantarum* M2 and *Leuc. pseudomesenteroides* I3 proved to be the most effective against *L. monocytogenes* and *Enterococcus* spp.

The culture medium pH after hydrolysate addition was approximately 6.6; thus, inhibition due to organic acid production can be excluded. Although the production of bacteriocins or antimicrobial exopolysaccharides cannot be excluded, the predominance of small peptides following fermentation suggests that these molecules may contribute to the observed antimicrobial activity. In particular, *Leuc. pseudomesenteroides* I3 consistently produced strong inhibitory effects, which may be linked to its ability to release the highest proportion of low-molecular-weight peptides (both 6–3 kDa and <3 kDa; Table 2.5), a feature often associated with effective antimicrobial activity (Liu et al., 2025). While *Lb. plantarum* M2 produced a lower overall peptide release, its fermentates still strongly inhibited Gram-positive growth. The literature shows that low-molecular-weight, cationic, and amphipathic peptides are generally more active because they can interact with bacterial membranes (Elsaadany et al., 2024; Olvera-Rosales et al., 2023). The reduction in the maximum growth rate and final biomass observed here suggests that peptide activity not only delayed adaptation but also impaired cellular metabolism and limited biomass accumulation. This observation is consistent with membrane disruption and possible interference with intracellular processes, such as DNA replication, reported for pea-derived peptides (Wang et al., 2025).

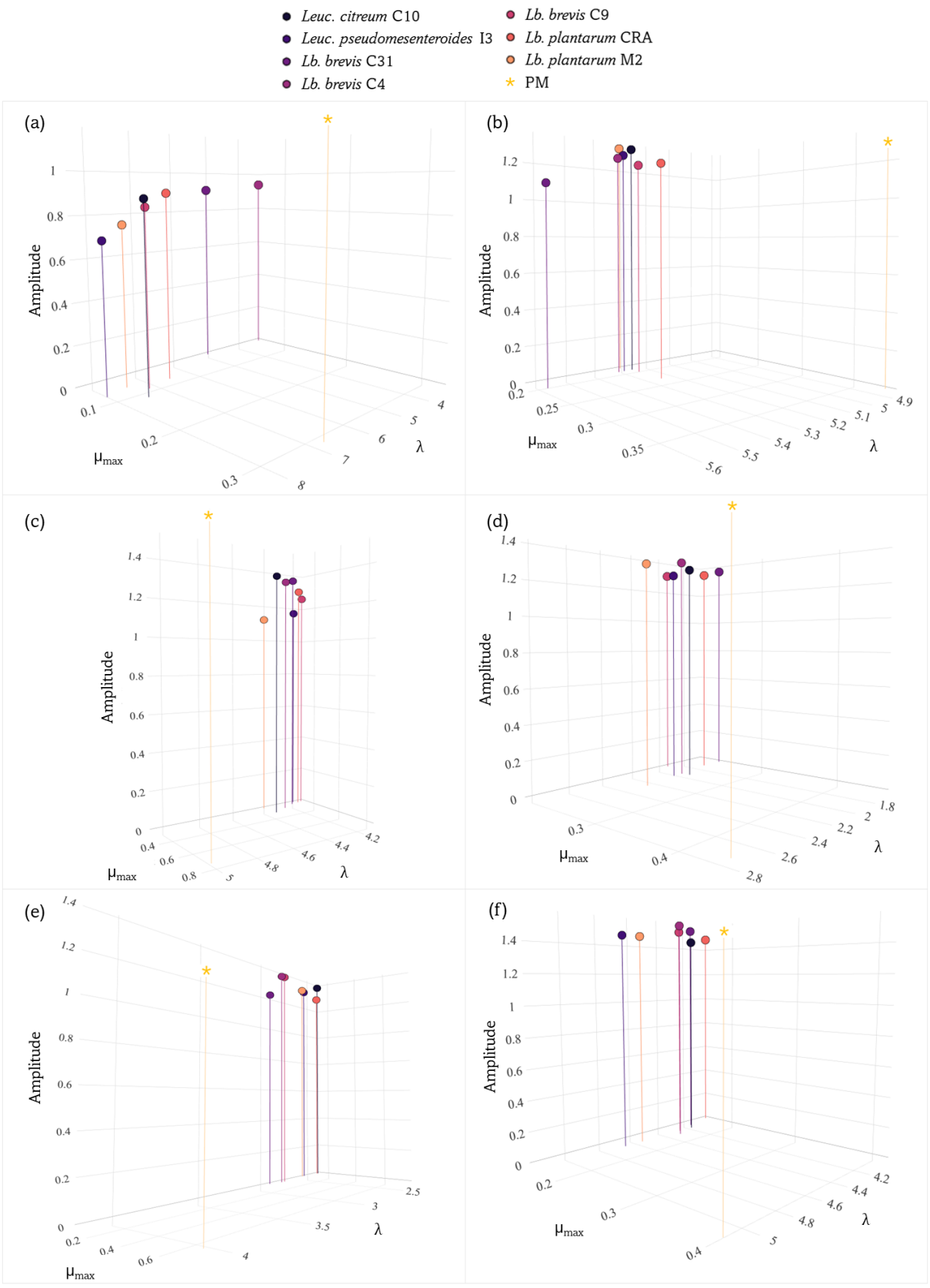

Figure 4.3 Three-dimensional scatterplots showing the effect of the fermentates and the unfermented pea medium (PM) on the growth kinetics of (a) *L. monocytogenes*, (b) *Staph. aureus*, (c) *Enterococcus* spp., (d) *E. coli*, (e) *Salm. enterica*, and (f) *Ps. fluorescens*. The plots represent the fitted growth parameters estimated by the re-parametrised Gompertz model, where Amplitude corresponds to the upper asymptote of the growth curve, μ_{max} is the maximum specific growth rate (log OD/h), and λ represents the lag time (h)

Notably, *Lb. plantarum* M2 combined antimicrobial efficacy with marked antioxidant properties, underscoring its potential to generate fermentates with complementary bioactivities (Raveschot et al., 2018).

In contrast, no apparent antimicrobial effect was observed against Gram-negative bacteria. Fermentation often led to a significantly shorter λ and higher A, indicating that growth was favored despite a general reduction in μ_{max} ($p<0.05$). This trend was consistent across all three Gram-negative targets tested. The only notable exception was, once again, the fermentate produced by *Leuc. pseudomesenteroides* I3, which, although associated with a slightly shorter λ, caused a reduction in both the μ_{max} and the final biomass of *E. coli* ($p<0.05$). This selective activity reflects the intrinsic resistance of Gram-negative bacteria, where the outer membrane acts as a barrier, reducing the effectiveness of many antimicrobial peptides (Mohan et al., 2019). Nevertheless, the *Leuc. pseudomesenteroides* I3 fermentate appears capable of overcoming this barrier to some extent, possibly due to its higher release of small cationic peptides, which are known to permeabilize membranes and, once internalized, interfere with essential cellular functions (Olvera-Rosales et al., 2023).

4.3.3 Impact on Gut Microbiota and Its Metabolites

Among the functional activities that pea proteins and their hydrolysates may exert, the ability to modulate the gut microbiota and the derived metabolites has recently gained attention. A recent study demonstrated that supplementation with pea-derived protein, combined with probiotics, altered gut microbial balance, favoring pathways of protein degradation and increasing plasma levels of essential amino acids (Mauricio Retuerto et al., 2023). However, to the best of our knowledge, no further studies have specifically addressed the direct impact of pea protein fermentates generated by LAB on the gut microbiota and microbial metabolites. The majority of soluble compounds released during protein digestion, such as small peptides and free amino acids, are absorbed in the upper intestine, where they may exert local functional effects, including antioxidant activity if they survive gastrointestinal transit. Nonetheless, it has been estimated that up to 10% of these compounds, along with the non-absorbed fraction, can reach the colon (Pérez-Burillo et al., 2021). This includes intact proteins, peptides, dietary fibers, and both live and dead microbial cells. All these components represent potential substrates for the colonic microbiota, thereby influencing its composition and metabolic activity. In light of this, the last part of this study was designed to investigate the impact of pea protein and its LAB-derived hydrolysates on the gut microbiota and its metabolites. The fermentates were first subjected to *in vitro* gastrointestinal digestion, monitoring microbial survival (Figure 4.4). Before gastrointestinal digestion (UND), all tested LAB strains exhibited high viability, ranging from approximately 7 to >8 log cfu/mL.

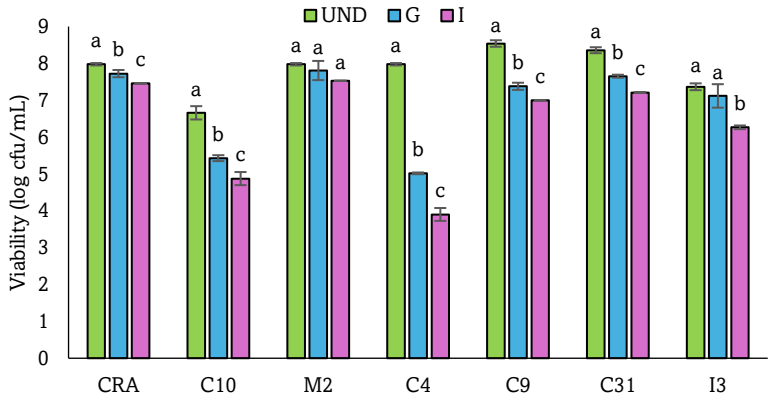

Figure 4.4 Viability (log cfu/mL; mean ± standard deviation) of bacterial strains during *in vitro* gastrointestinal digestion in the undigested sample (UND; green), after the gastric phase (G; blue), and after the intestinal phase (I; purple). Different letters indicate statistically significant differences among digestion phases within each strain (p<0.05)

Exposure to gastric and intestinal phases significantly affected survival, with strain-dependent differences. During the gastric phase (G), cells were exposed to harsh acidic conditions (pH ~3) and pepsin activity, mimicking the stomach environment (Castro-López et al., 2023). Despite these stresses, only limited reductions in viability (0-3 log cfu/mL reduction) were observed for most strains, indicating that the gastric phase had relatively little impact on their survival. This resilience is consistent with the acid tolerance commonly reported for LAB isolated from fermented plant matrices, where repeated exposure to acidic environments is a key selective pressure (Di Cagno et al., 2013). Accordingly, 5 out of 7 LAB strains maintained viability close to their initial levels. In contrast, *Lb. brevis* C4 and *Leuc. citreum* C10 exhibited greater sensitivity to acidic pH and pepsin digestion, displaying the sharpest decline during gastric exposure. The intestinal phase (I), characterized by the presence of bile salts and pancreatin (Castro-López et al., 2023), imposed another strong selective pressure, further reducing cell viability across almost all strains. *Lb. brevis* C4 and *Leuc. citreum* C10 showed the most significant reductions, dropping below 5 log cfu/mL, while *Lb. plantarum* CRA and M2 retained comparatively higher survival, remaining above 7 log cfu/mL. Notably, *Lb. brevis* C9 and C31, and *Leuc. pseudomesenteroides* I3 also showed relatively good survival, with all remaining at or above 7 log cfu/mL after digestion, despite significant reductions from their initial levels. Overall, several strains demonstrated the ability to survive gastrointestinal transit with high viable counts, underlining their potential for functional food applications.

The undigested fractions were then used as substrates in fecal batch fermentations, during which changes in microbiota composition were assessed by 16S rRNA gene sequencing. Figure S4.1 illustrates the relative abundance of the top 25 taxa after fecal fermentation of unfermented PM and of the fermentates obtained with the different LAB strains. The sequencing coverage enabled the identification of nearly 100% of the microbial species present in the samples, confirming the robustness of the analysis. Overall, the community profiles shared similar core structures. *Phascolarctobacterium faecium* was one of the most abundant taxa across all fermentations. This species

is a succinate-utilizing bacterium whose growth is supported by cross-feeding interactions with *Bacteroides* spp., major producers of succinate in the gut (Ikeyama et al., 2020). The concurrent high abundance of *Bacteroides* observed in our samples is therefore consistent with the substantial representation of *Phascolarctobacterium faecium*, and together these taxa likely contributed to the production of propionate and acetate, SCFAs with well-established beneficial effects on host metabolism and gut health (Ikeyama et al., 2020; Verma et al., 2024).

Among the *Bacteroides* genus, *Bacteroides vulgatus* was consistently detected at high relative abundance. This species is commonly associated with protein-based substrates and has been linked to adverse effects when overrepresented, including inflammatory conditions like ulcerative colitis (Mills et al., 2022). *Bacteroides massiliensis* was also among the dominant species, but is less frequently reported in gut studies. Alongside these species, *Bilophila wadsworthia* was also consistently detected. This sulfite-reducing bacterium is often associated with high-protein or high-fat diets and produces hydrogen sulfide, a metabolite with pro-inflammatory potential and links to gut barrier disruption (Feng et al., 2017).

In contrast, *Bifidobacterium longum* was also found consistently. It is recognized as a probiotic species capable of effectively colonizing the human gut, where it can maintain stable populations over time. Beyond its persistence, this species exerts multiple health-promoting functions: it ferments complex dietary carbohydrates, leading to the production of acetate and lactate; it contributes to the inhibition of pathogenic microorganisms through competitive exclusion and acidification of the environment; and it engages in cross-feeding interactions by providing substrates that support the growth of butyrate-producing bacteria (Zhang et al., 2019). These dominant taxa accounted for the majority of the community, while other genera were detected at lower relative abundance.

To gain deeper insight into the biodiversity of the microbial communities that developed, alpha diversity indices were computed for the PM and the hydrolysates following *in vitro* colonic fermentation (Table 4.6).

Table 4.6 Alpha diversity indices (Richness, Simpson, and Shannon) of microbial communities detected in pea medium (PM) and in the fermentates after in vitro colonic fermentation

Sample	Number of taxa	Simpson index	Shannon index
PM	20	0.85	2.46
Lb. plantarum CRA	18	0.85	2.43
Leuc. citreum C10	21	0.86	2.51
Lb. plantarum M2	19	0.84	2.40
Lb. brevis C4	19	0.85	2.40
Lb. brevis C9	21	0.87	2.55
Lb. brevis C31	18	0.83	2.27
Leuc. pseudomesenteroides I3	19	0.84	2.37

These indices offer different yet complementary perspectives on the microbial community structure. The number of taxa represents the species richness within a sample. In contrast, the Shannon and Simpson indices account for the distribution of individuals among those species, thereby capturing aspects of community balance, evenness, and dominance (Hill et al., 2003). The number of taxa ranged between 18 and 21 across samples, suggesting similar species richness. Among the fermentates, *Lb. brevis* C9 and *Leuc. citreum* C10 displayed higher values for both the Simpson and Shannon indices, reflecting a more balanced and diverse community structure. Conversely, *Lb. brevis* C31 and *Leuc. pseudomesenteroides* I3 showed lower diversity, indicating greater dominance of specific taxa.

To better characterize the variation in microbial community profiles among treatments, PCA was performed (Figure 4.5). The first two components (PC1 and PC2) together accounted for the majority of the dataset's variance, with PC1 explaining 41 % and PC2 explaining 26 %.

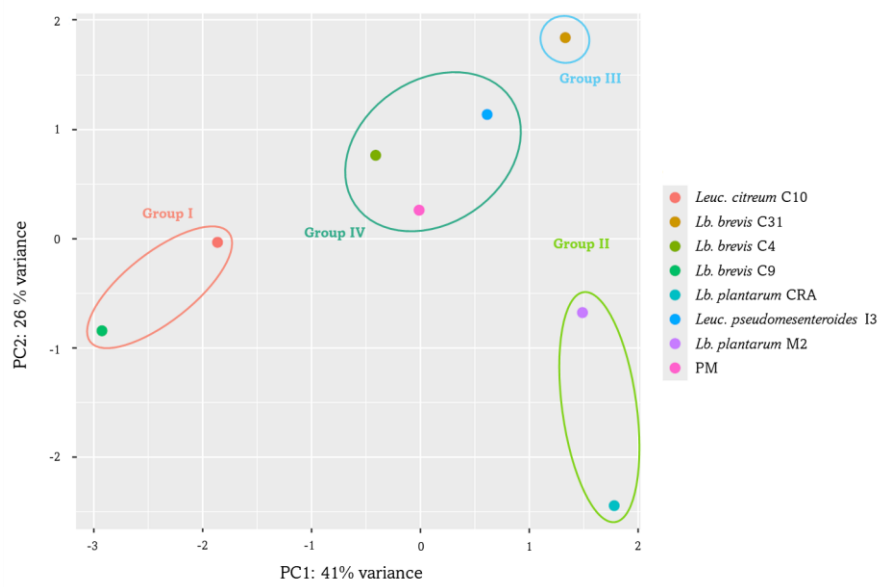

Figure 4.5 Principal component analysis (PCA) biplot for microbial species modulation by *in vitro* colonic fermentation of pea medium (PM) and fermentates. Samples are grouped according to similarity in microbial composition, as identified by hierarchical clustering and heatmap analysis

In parallel, hierarchical clustering was conducted, and the results are displayed as a heatmap with dendrograms (Figure S4.2). Both approaches consistently indicated that the samples grouped into four main clusters, reflecting distinct patterns of microbial modulation in response to the different fermentates (Figure 4.5). Group I, represented by the fermentates obtained with *Leuc. citreum* C10 and *Lb. brevis* C9 showed the most positive modulation. These samples were associated with an enrichment of saccharolytic and SCFA-producing taxa such as *Bacteroides uniformis*, *Bacteroides stercoris*, and *Bifidobacterium longum* (Fabersani et al., 2021; Zhang et al., 2019), suggesting that the fermentates generated by these strains promoted a beneficial microbial environment with enhanced activity. Group II, including *Lb. plantarum* CRA and M2, also showed a shift toward

communities enriched in beneficial commensals, such as *Blautia spp.*, *Anaerostipes hadrus*, and *Fusicatenibacter saccharivorans*, all recognized for their roles in SCFA production (Liu et al., 2024; Liu et al., 2021). However, this group also showed the presence of some taxa associated with less favorable microbial profiles, indicating a more heterogeneous and moderate modulatory effect. Group IV included the unfermented PM together with the fermentates from *Lb. brevis* C4 and *Leuc. pseudomesenteroides* I3. This cluster indicates that the corresponding hydrolysates induced only minor changes in the fecal microbiota compared to the PM, maintaining a composition comparable to that of the unfermented substrate. Group III, represented by the fermentate from *Lb. brevis* C31 was positioned in a distinct area of the PCA plot, within the same quadrant as Group IV, suggesting a degree of similarity in the overall community structure compared to the unfermented PM.

To gain deeper insight into the modulatory effects of LAB fermentates, we employed the $\log_2$ fold change parameter to compare their impact on microbial composition with that of the unfermented PM (Table 4.7). This approach quantifies how the relative abundance of each taxon shifts in the presence of the hydrolysates, with positive values indicating enrichment and negative values indicating a reduction compared to the unfermented PM. To contextualize these changes, taxa were further categorized into three broad functional groups: (i) probiotics or next-generation probiotics, (ii) health-supporting commensals, and (iii) disease and dysbiosis-associated microbes. Such classification provides a clearer framework to interpret whether the observed modulations reflect beneficial or detrimental trends in the microbial community following exposure to pea protein hydrolysates. A negative outcome was observed for *Bifidobacterium longum*, whose abundance decreased in the fecal fermentation of products derived from *Lb. plantarum* CRA and *Lb. brevis* C31. Given the well-established probiotic role of this species in the gut, such a reduction may be considered undesirable. Similarly, several health-supporting commensals, including *Odoribacter splanchnicus*, *Bacteroides uniformis*, and *Adlercreutzia equolifaciens*, were reduced after colonic fermentation of the hydrolysates compared to the PM control.

Nevertheless, some positive modulations were also evident. Fermentates from *Lb. brevis* C9 and *Leuc. citreum* C10 promoted the growth of *Parabacteroides distasonis*, a species increasingly recognized for its health-promoting properties, including anti-inflammatory potential and contribution to metabolic homeostasis (Wang et al., 2019). In addition, several fermentates (*Lb. plantarum* CRA and M2, *Leuc. citreum* C10, *Lb. brevis* C4 and C9, and *Leuc. pseudomesenteroides* I3) led to reduced abundance of opportunistic pathogens or microbes associated with dysbiosis, such as *Bilophila wadsworthia*, *Parasutterella excrementihominis*, and *Dialister invisus*. The reduction of *Bilophila wadsworthia* is particularly relevant, since it has been linked to pro-inflammatory processes and colonic barrier dysfunction (Feng et al., 2017), and it represented one of the dominant species across all samples (Figure S4.1).

Table 4.7 Log_2 fold change (log_2PM/fermented sample) in relative abundance of selected bacterial species after *in vitro* fecal fermentation with different LAB fermentates. Only species with $p<0.05$ are reported. Positive values indicate a relative increase in the fermented sample compared to the unfermented pea medium (PM), whereas negative values indicate a relative decrease. Species are grouped according to functional categories: probiotics and next-generation probiotics, health-supporting commensals, and disease- or dysbiosis-associated microbes

Species	*Lb. plantarum* CRA	*Leuc. citreum* C10	*Lb. plantarum* M2	*Lb. brevis* C4	*Lb. brevis* C9	*Lb. brevis* C31	*Leuc. pseudomesenteroides* I3
Probiotics and Next Generation Probiotics							
Bifidobacterium longum	-0.4	-	-	-	-	-0.68	-
Health-supporting commensals							
Phascolarctobacterium faecium	-	-	-	-	-	0.32	-
Ruminococcus bromii	-	-	-	-	-	-0.59	-
Odoribacter splanchnicus	-1.43	-	-1.71	-	-	-	-
Parabacteroides distasonis	-0.31	0.48	-0.38	-0.34	0.56	-	-
Bacteroides uniformis	-0.78	-	-0.43	-	-	-0.28	-
Adlercreutzia equolifaciens	-6.06	-	-	-	-	-	-
Parabacteroides merdae	-1.48	-	-	-	-	-	-
Disease and dysbiosis-associated microbes							
Bilophila wadsworthia	-0.28	-0.23	-0.51	-0.21	-	-0.39	-
Parasutterella excrementihominis	-	-0.31	-	-0.49	0.49	-0.75	-
Dialister invisus	-0.9	-	-0.59	-	-0.64	-	-
Veillonella parvula	2.34	-	1.88	-	-	-	-
Haemophilus parainfluenzae	-5.77	-	-	-	-	-	-

The decrease in *Parasutterella excrementihominis* is also noteworthy, as this taxon has been associated with intestinal disorders and progression of liver disease (Galley et al., 2024). Likewise, the reduced representation of *Dialister invisus*, a taxon often found enriched in inflammatory conditions and metabolic disorders (Zhang et al., 2025a), may be interpreted as a beneficial outcome. On the other hand, *Veillonella parvula* was promoted in the fecal fermentations of *Lb. plantarum* CRA and M2 hydrolysates. This species is a lactate-utilizing bacterium and has recently been linked to metabolic alterations and opportunistic pathogenicity (Cobo et al., 2024), thus, its enrichment may raise concerns. Conversely, fermentate derived from *Lb. plantarum* CRA also led to a marked reduction of *Haemophilus parainfluenzae*, a bacterium associated with respiratory infections and invasive disease in humans (Oill et al., 1979), which can be considered a favorable effect.

The colonic fermentation of pea protein hydrolysates was accompanied by the modulation of SCFAs and BCFAs, reflecting the overall metabolic activity of the microbial community. SCFAs represent central end-products of carbohydrate and peptide fermentation, with recognized roles in energy harvesting, intestinal homeostasis, and signaling to host immune and metabolic pathways (Verma et al., 2024). The SCFA profile across samples was dominated by acetate, followed by propionate, formate, and, at lower concentrations, butyrate (Table 4.8). The predominance of acetate in our samples is consistent with recent evidence indicating that acetate is the major SCFA produced in the human gut following fecal fermentation of pea protein. At the same time, propionate and butyrate were present at lower levels (Karlsson et al., 2024). After colonic fermentation of PM, acetate reached 41.74 ± 1.32 mM, whereas in most other samples it was reduced. As the predominant SCFA in the colon, acetate plays a central role in host energy metabolism and lipid regulation, and its reduction may indicate a negative shift in microbial fermentative activity.

A similar trend was observed for formic acid (12.78 ± 0.45 mM in PM), although reductions were less pronounced: only *Lb. plantarum* CRA (11.57 ± 0.23 mM) and *Lb. brevis* C31 (12.00 ± 0.33 mM) showed significant decreases. Formate, despite its lower abundance, remains relevant as an intermediate in microbial cross-feeding interactions. In contrast, propionic acid levels were generally higher than in PM (17.69 ± 0.15 mM), with the strongest enrichment in *Leuc. citreum* C10 (23.20 ± 0.12 mM), followed by *Lb. brevis* C4 (19.25 ± 0.08 mM) and *Lb. plantarum* CRA (20.73 ± 0.01 mM). Propionate is considered a beneficial metabolite due to its role in glucose homeostasis and its recognized immunomodulatory effects. Butyric acid, although present at low concentrations in PM (1.45 ± 0.04 mM), was depleted in most fermentates.

Table 4.8 Concentrations of short-chain fatty acids (SCFA, mM) and branched-chain fatty acids (BCFA, mM) produced during *in vitro* fecal fermentation of the undigested fraction of pea medium (PM) and of protein hydrolysates obtained from pea protein by fermentation with selected strains. Data are expressed as mean ± standard deviation

	SCFA (mM)				BCFA (mM)	
	Formic acid	Acetic acid	Propionic acid	Butyric acid	Isobutyric acid	Isovaleric acid
PM	$12.78^{a} \pm 0.45$	$41.74^{a} \pm 1.32$	$17.69^{b} \pm 0.15$	$1.45^{a} \pm 0.04$	$0.06^{d} \pm 0.01$	$3.58^{a} \pm 0.07$
Lb. plantarum CRA	$11.57^{b} \pm 0.23$	$33.09^{c} \pm 0.31$	$20.73^{a} \pm 0.01$	n.d.	$5.25^{a} \pm 0.18$	$3.76^{a} \pm 0.58$
Leuc. citreum C10	$12.34^{ab} \pm 0.07$	$39.23^{b} \pm 0.15$	$23.20^{a} \pm 0.12$	n.d.	$0.44^{d} \pm 0.20$	$4.33^{a} \pm 0.54$
Lb. plantarum M2	$13.29^{a} \pm 0.23$	$39.48^{b} \pm 0.39$	$16.62^{b} \pm 0.31$	$1.89^{a} \pm 0.21$	$3.21^{b} \pm 0.16$	$3.28^{a} \pm 0.25$
Lb. brevis C4	$12.97^{a} \pm 0.02$	$44.58^{a} \pm 0.24$	$19.25^{a} \pm 0.08$	n.d.	$0.69^{c} \pm 0.07$	$3.47^{a} \pm 0.10$
Lb. brevis C9	$12.84^{a} \pm 0.13$	$40.55^{b} \pm 1.81$	$20.73^{ab} \pm 3.24$	n.d.	$0.66^{c} \pm 0.06$	$4.07^{a} \pm 0.62$
Lb. brevis C31	$12.00^{b} \pm 0.33$	$43.81^{a} \pm 0.83$	$18.61^{b} \pm 0.23$	n.d.	$0.82^{c} \pm 0.08$	$3.2^{a} \pm 0.13$
Leuc. pseudomesenteroides I3	$13.21^{a} \pm 0.21$	$44.12^{a} \pm 0.49$	$17.46^{b} \pm 0.29$	$1.52^{a} \pm 0.17$	$0.95^{c} \pm 0.13$	$3.03^{a} \pm 0.17$

Statistical differences ($p<0.05$) within each column are indicated by different letters

n.d. not detected

Spearman's correlation analysis highlighted several associations between microbial taxa and butyric acid (Figure 4.6). It was positively associated with *Blautia luti* and, although not statistically significant, also with *Blautia massiliensis* and *Anaerostipes hadrus*, species well known for their butyrogenic activity. Similarly, formic acid showed positive associations with *Blautia* spp., suggesting cross-feeding interactions within the microbial network. Nevertheless, in many other fermentates, both butyrate and formate concentrations declined compared to these samples, indicating that not all hydrolysates were equally effective in maintaining these health-relevant metabolic outputs.

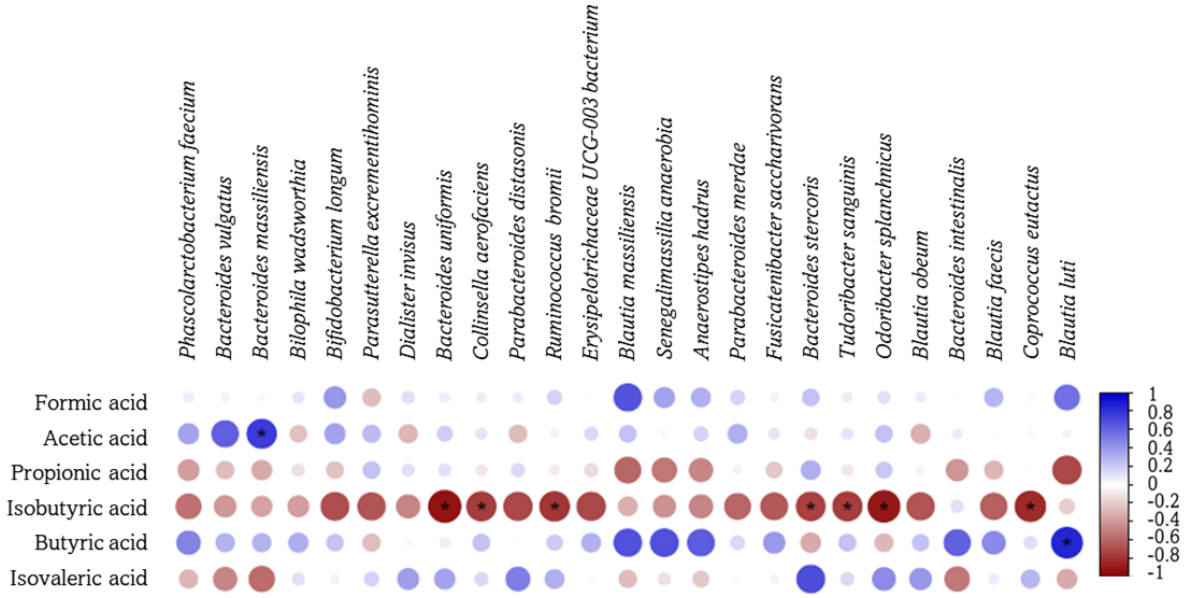

Figure 4.6 Spearman's correlation heatmap between the top 25 microbial taxa for relative abundance (columns) and the concentration of short- and branched-chain fatty acids (SCFA and BCFA) (rows). Positive correlations are shown in blue; negative correlations are shown in red. The circle dimension is proportional to the absolute value of Spearman's correlation coefficient. Significant coefficients (p<0.05) are denoted by an asterisk (*)

BCFAs arise mainly from the proteolytic metabolism of branched-chain amino acids. While traditionally regarded as markers of protein fermentation, recent studies have highlighted that these metabolites may also play potentially beneficial roles in host physiology (Rios-Covian et al., 2020). Isovaleric acid remained relatively stable across conditions, showing no sample-dependent differences (Table 4.8). Conversely, isobutyric acid, though produced at much lower levels, displayed the most variable pattern. While PM contained only 0.06 ± 0.01 mM, all fermentates promoted its enrichment, with particularly marked increases in *Lb. plantarum* CRA (5.25 ± 0.18 mM) and *Lb. plantarum* M2 (3.21 ± 0.16 mM). Chi et al. (2024) reported that BCFA, including isobutyrate, improved intestinal development and barrier function in piglets. In our study, however, correlation analysis revealed that isobutyrate was negatively associated with several beneficial commensals, including *Bacteroides uniformis*, *Ruminococcus bromii*, *Odoribacter splanchnicus*, and *Coprococcus eutactus* (Figure 4.6). The reduction of these health-supporting taxa is generally undesirable, as they play essential roles in maintaining gut homeostasis. For instance, *Ruminococcus bromii* is a keystone species in the degradation of resistant starch and a major contributor to butyrate production

(Loaiza et al., 2025), while *Coprococcus eutactus* has been linked to positive effects on host neurophysiology and attenuation of stress-related behaviors (Xu et al., 2024). By contrast, the negative associations observed for *Tudoribacter sanguinis* and *Collinsella aerofaciens* may represent a more favorable outcome, as these taxa have been linked to pro-inflammatory activity and dysbiosis (Cuenca et al., 2022; Kulkarni et al., 2021).

The fermentates generated by LAB are inherently complex substrates, containing residual carbohydrates, microbial metabolites, peptides, free amino acids, and partially hydrolyzed proteins that may, in part, reach the colon. Thus, to investigate whether the biochemical profiles of the protein hydrolysates, measured before *in vitro* digestion and fecal fermentation, influenced subsequent modulation of the gut microbiota, correlation analyses were performed. Lactic acid was negatively associated with *Bacteroides* species (p<0.05), particularly *Bacteroides vulgatus* (Figure 4.7).

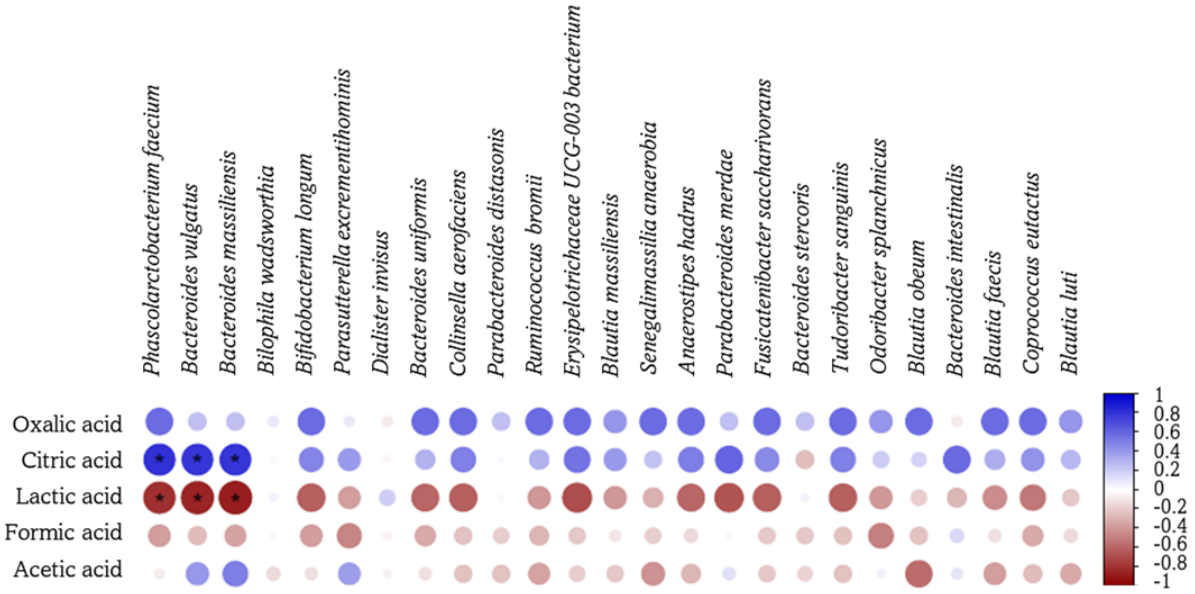

Figure 4.7 Spearman's correlation heatmap between the top 25 microbial taxa for relative abundance (columns) and the concentration of organic acids detected in the hydrolysates before *in vitro* digestion and colonic fermentation (rows). Positive correlations are shown in blue; negative correlations are shown in red. The circle dimension is proportional to the absolute value of Spearman's correlation coefficient. Significant coefficients (p<0.05) are denoted by an asterisk (*)

This finding is noteworthy, as lactic acid was consistently enriched in all hydrolysates due to lactic fermentation, and the reduction of *Bacteroides vulgatus* can be considered a favorable effect. On the other hand, lactic acid also showed negative associations with beneficial commensals such as *Phascolarctobacterium faecium*. These contrasting associations suggest that the effects of lactic acid accumulation may be context-dependent, promoting reductions in undesirable taxa while simultaneously affecting health-supporting members of the community. Citrate displayed positive correlations with some microorganisms, including *Bacteroides vulgatus*. However, since citric acid was detected only at trace levels or was absent in most samples, its role in shaping microbial responses in this experimental setting is likely negligible.

Finally, correlations were evaluated with the nitrogen component of the fermentates. While no significant associations were detected between peptide abundance and microbial taxa, several amino acids showed relevant correlations (Figure 4.8).

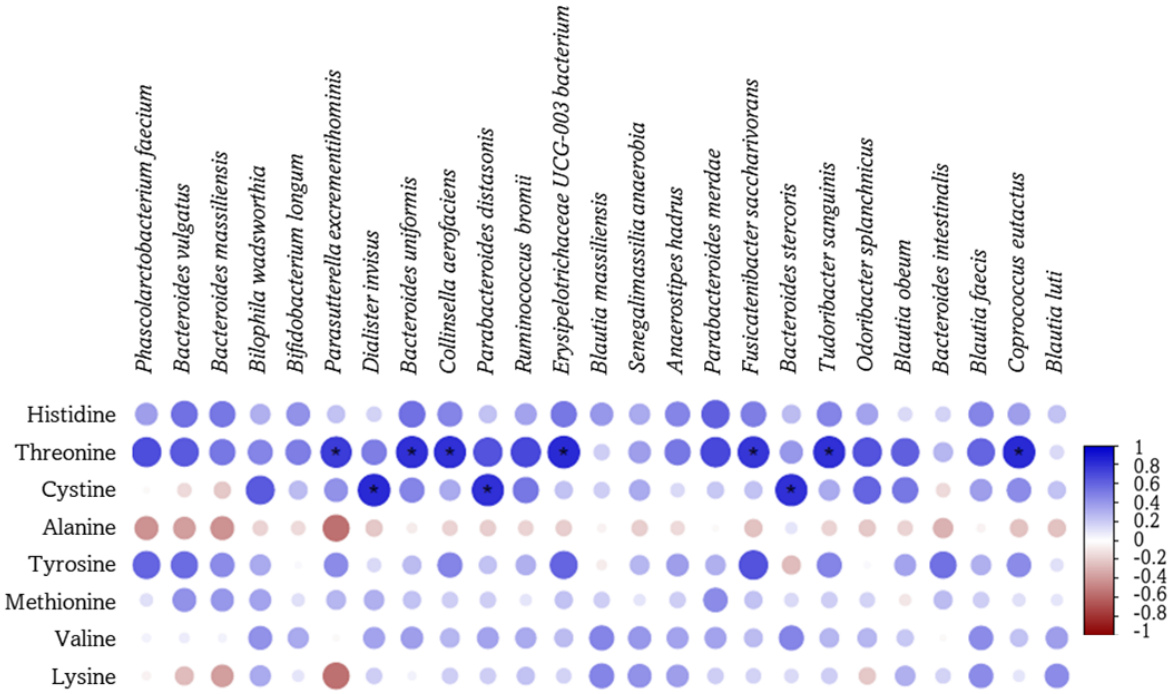

Figure 4.8 Spearman's correlation heatmap between the top 25 microbial taxa for relative abundance (columns) and the concentration of amino acids detected in the hydrolysates before *in vitro* digestion and colonic fermentation (rows). Positive correlations are shown in blue; negative correlations are shown in red. The circle dimension is proportional to the absolute value of Spearman's correlation coefficient. Significant coefficients (p<0.05) are denoted by an asterisk (*)

Overall, most amino acids, except alanine, showed positive associations with both beneficial and potentially adverse taxa, although not all reached significance. Among the statistically significant findings, threonine was positively correlated (p<0.05) with health-associated species, including *Bacteroides uniformis*, *Fusicatenibacter saccharivorans*, and *Coprococcus eutactus* (Xu et al., 2024). Interestingly, threonine was also positively linked with an *Erysipelotrichaceae bacterium* (p<0.05), a taxon often associated with protein fermentation; this observation aligns with recent findings that amino acid metabolism, including N-acetylgalactosamine and related substrates, plays a central role in shaping the metabolic activity of *Erysipelotrichaceae* strains, with positive effects on the overall nitrogen turnover and metabolic diversity in the gut (Zhou et al., 2024). Furthermore, cystine showed positive correlations (p<0.05) with beneficial commensals, including *Parabacteroides distasonis* and *Bacteroides stercoris*. Still, it was also associated with *Dialister invisus*, a species commonly linked to unfavorable fermentative profiles.

4.4 Conclusions

The results of this study demonstrate that LAB fermentation of pea protein is highly strain-dependent, with distinct consequences for product functionality and potential health benefits. Strains

differed in their carbohydrate metabolism, producing distinct organic acid profiles that affected acidification and potentially sensory qualities, while their proteolytic activities generated peptide mixtures of different composition. These strain-dependent features were directly linked to biofunctional outcomes. The fermentate obtained with *Lb. brevis* C9 exhibited the strongest radical-scavenging capacity, while fermentate *Lb. plantarum* M2 showed the highest reducing power and, together with *Leuc. pseudomesenteroides* I3, consistent antimicrobial effects against Gram-positive bacteria. At the gut level, some fermentates supported favorable modulation by promoting health-associated taxa or reducing species linked to dysbiosis. However, in other cases, the modulation was less optimal, with reductions of beneficial microbes or enrichment of taxa with potentially negative implications. Notably, *Leuc. citreum* C10 emerged as the hydrolysate with the most consistent positive impact on microbial composition, favoring probiotic and commensal taxa such as *Bifidobacterium longum* and *Parabacteroides distasonis*, while reducing pro-inflammatory species like *Bilophila wadsworthia*.

In contrast, fermentate derived from *Lb. plantarum* M2 was particularly effective in sustaining beneficial microbial metabolites, such as butyrate and formate, although it also promoted the growth of less desirable taxa. Overall, the results underscore the importance of selecting LAB not only for their growth performance or proteolytic capacity, but also for their ability to generate metabolites with biofunctional properties and to drive favorable shifts in the gut microbiota. Their careful selection may offer a strategy for designing pea-based fermented beverages with enhanced health-promoting potential.

4.5 Supplementary Material

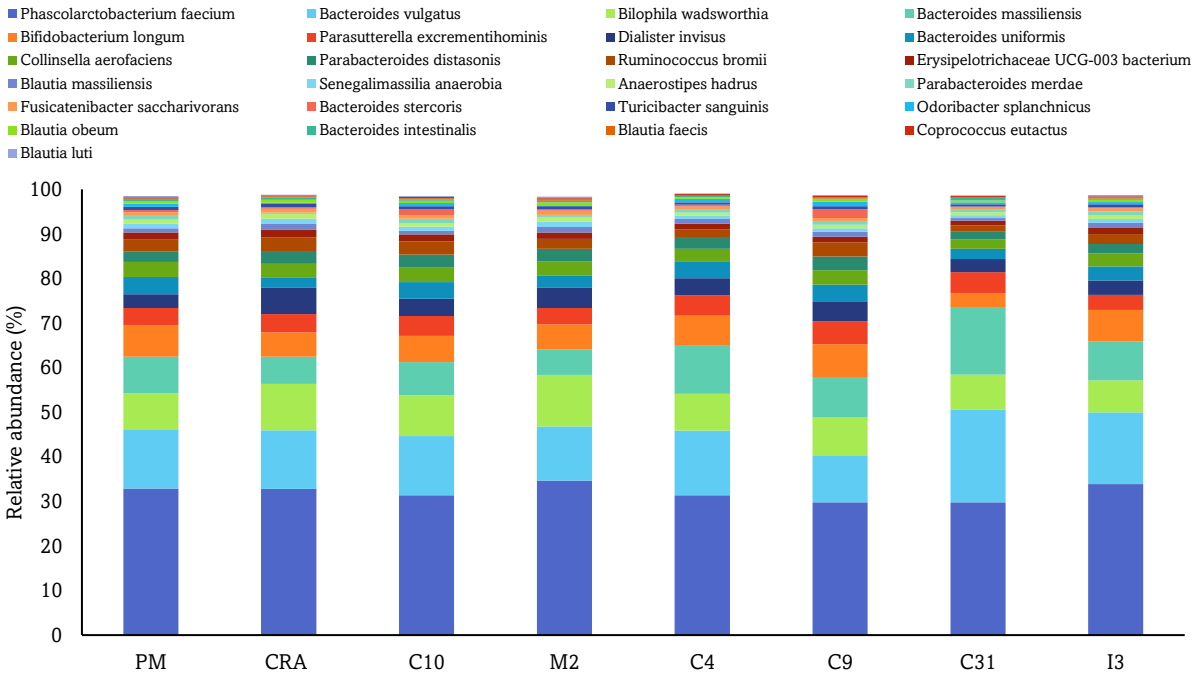

Figure S4.1 Relative abundance (%) of top 25 microbial taxa found in the colonic fermented samples of unfermented pea medium (PM) and LAB fermentates

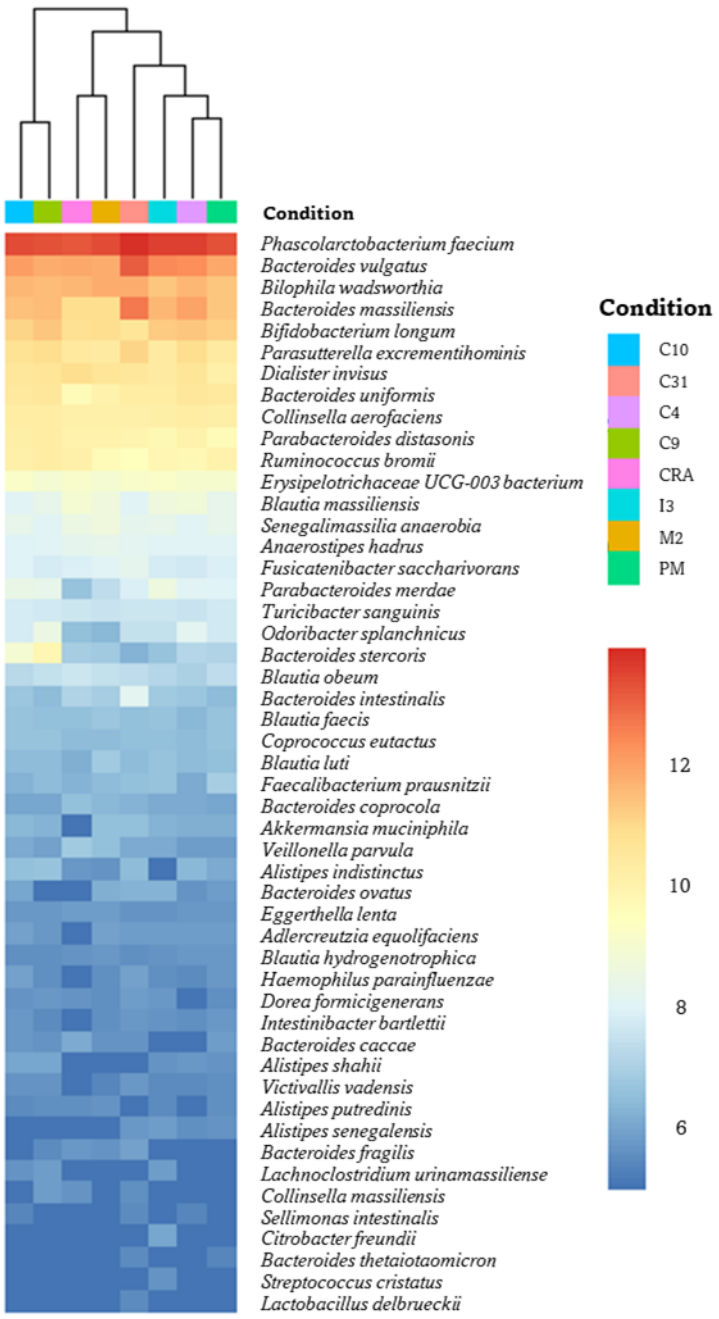

Figure S4.2 Heatmap of microbial community composition after *in vitro* fecal fermentation of unfermented pea medium (PM) and LAB fermentates. Samples are clustered according to similarity in taxonomic profiles, with relative abundances of predominant taxa represented by the color scale

Chapter 5:
Proteomics Insight into the Role of LAB Fermentation in Whey and Pea Protein Valorization

5.1 Introduction

Lactic acid bacteria (LAB) represent a diverse group of Gram-positive microorganisms widely exploited in the food industry to produce fermented foods (FFs), including dairy products, sourdoughs, cured meats, and fermented vegetables. Their importance lies not only in their ability to shape sensory traits and the product's shelf life during fermentation, but also in their capacity to act as probiotics and generate bioactive compounds that contribute to human health (Abedin et al., 2024; Ter et al., 2024). Within this group, the genus *Lactobacillus* comprises metabolically versatile strains that can adapt to diverse ecological niches. *Lacticaseibacillus paracasei* stands out for its remarkable adaptability and probiotic potential, making it a valuable candidate for designing fermented products from both animal- and plant-based matrices (Grujović et al., 2022). This flexibility is particularly relevant given the increasing demand for sustainable protein sources. Plant proteins, such as those from peas, combined with the valorization of nutrient-rich by-products, like cheese whey, represent complementary strategies for developing a sustainable food system. Their use, alone or in combination, not only reduces environmental impact and food waste but also provides promising substrates that, through fermentation, can be transformed into FFs with enhanced health-promoting properties (Kuo et al., 2024; Sabater et al., 2020).

In protein-based substrates, *Lb. paracasei* relies heavily on proteolysis to recover amino acids necessary for growth. Its activity may release a diverse array of peptides with potential bioactivities (Pessione & Cirrincione, 2016; Savijoki et al., 2006). However, proteolysis is only one of several metabolic pathways active during fermentation: carbohydrate metabolism, amino acid catabolism, and other microbial processes produce a diverse array of metabolites, collectively shaping the complex fermentate rich in compounds that act individually or synergistically to confer nutritional, technological, and health-promoting properties (Ter et al., 2024).

Since microbial metabolism is strongly influenced by growth media, understanding how *Lb. paracasei* adapts to different substrates is crucial for optimizing the fermentation process and maximizing the functional potential of the products. During fermentation, LAB adapt to variable environmental conditions, including different available sugars, pH, and other media components. By modulating their metabolism, LAB alter the end metabolites produced, resulting in distinct profiles of bioactive compounds (Li et al., 2021b; Siragusa et al., 2014).

Proteomics, defined as the large-scale characterization of the entire proteins expressed by a single microbe or a microbial community, is a powerful tool for unraveling microbial metabolic responses and adaptation (Van Den Bossche et al., 2025). To date, only a few studies have applied proteomics to investigate the metabolic adaptation of *Lb. paracasei* across different growth conditions. Most of this research has been carried out in laboratory media, often modifying specific parameters such as carbon source availability or stress conditions. Still, such environments differ substantially from

those encountered during real food fermentations (Siragusa et al., 2014). Studies exploring how *Lb. paracasei* adapts to food substrates, particularly protein-based matrices of plant and animal origin, remain scarce. Wang et al. (2013) showed that *Lacticaseibacillus casei* Zhang grew faster in soy beverage than in milk and, through proteomic analysis across three growth phases, revealed substrate-specific variations in protein expression that accounted for physiological differences. Despite these insights, no proteomic investigations have been conducted to valorize alternative protein-based resources with a focus on health-promoting outcomes.

To address this gap, the present study investigated the metabolic adaptation of a single *Lb. paracasei* strain in three protein-based substrates - pea, whey, and a combination of the two - used as models for plant-based, animal-based, and mixed fermented beverages. Proteomic analyses were integrated with analytical measurements of sugars, organic acids, amino acids, and proteolytic activity, while the bioactive properties of the fermentates were assessed. This approach aimed to elucidate the relationships among overall metabolic processes, proteolysis, and the characterization of functional properties in fermented products.

5.2 Materials and Methods

5.2.1 Materials

Ringer's solution, MRS broth, and bacteriological agar were purchased from Oxoid (Milan, Italy). Glycerol, tris-HCl, sodium dodecyl sulfate, dithiothreitol, iodoacetamide, methanol, phosphoric acid, ammonium bicarbonate, trypsin, trifluoroacetic acid (TFA), acetonitrile, and formic acid were obtained from Merck (Darmstadt, Germany). Other materials used are listed in Sections 2.2.1 and 3.2.1.

5.2.2 Media Preparation and Culture Conditions

Whey medium (WM) and pea medium (PM) were prepared according to Sections 2.2.5.2 and 2.2.6.2, respectively. Additionally, three different combinations of WM and PM were prepared with the same procedure in the following volumetric ratios: 50:50 (WPM), 75:25 (WPM 75:25), and 25:75 (WPM 25:75).

Lb. paracasei AF43, isolated from natural raw sheep's milk cheese and selected for proteolytic activity in Chapter 2, was stored at − 80 °C in MRS broth with 30 % glycerol (v/v). Before each use, the strain was reactivated in 1 mL of MRS at 30 °C for 24 h. Then, an overnight culture was prepared by inoculating 1 mL of MRS broth with 10 µL of the reactivated culture and incubating it at 30 °C for 24 h. The culture was then centrifuged at 13,000 × g for 3 min using a Mikro 20 centrifuge (Hettich Italia S.r.l., Milan, Italy). The resulting pellet was washed three times and diluted 1:100 with Ringer's solution.

5.2.3 Determination of Growth Kinetics

For determining growth kinetics, 96-well U-bottom microplates (Corning Life Sciences, Corning, NY, USA) were used. Each well was filled with 180 µL of each medium and 20 µL of the culture (final concentration approximately 10^6 cfu/mL). Control wells containing inoculated MRS broth and blank wells containing uninoculated media were also prepared. Microbial growth was monitored using a Sunrise microplate reader (Tecan, Milan, Italy) set at 30 °C, measuring optical density at 630 nm every 30 min after a 5 s shaking step. Experiments were carried out in triplicate. The elaboration of growth curves using the online tool DMfit (combase-browser.errc.ars.usda.gov/DMFit.aspx) enabled the determination of the time required to reach the mid-exponential and stationary phases.

5.2.4 PM, WM, and WPM Fermentation

The *Lb. paracasei* AF43 culture was inoculated in PM, WM, and WPM (final concentration approximately 10^6 cfu/mL) and incubated at 30 °C. Fermentation progress was monitored at three time points: the start (start), the mid-exponential phase (mid-exp), and the stationary phase (stat), identified from the corresponding growth curves. At each sampling point, pH was measured using a 5012T pH meter (Crison, Barcelona, Spain), and bacterial counts were determined by the drop-plate method (Herigstad et al., 2001) on MRS agar plates, which were incubated anaerobically at 30 °C for 48 h. Additionally, samples from mid-exp and stat phases were collected and centrifuged at 5,000 × g for 5 min at 4 °C. The resulting pellet was washed twice with Ringer's solution (5,000 × g, 5 min, 4°C) and used for proteomic analysis, while the supernatant was collected for metabolite profiling and functional activities evaluation.

5.2.5 Characterization of the Fermentates

The fermentation supernatants from the stat phase were analyzed for sugars and organic acids (as described in Section 3.2.3.2), free amino acids (Section 3.2.3.3), and the extent of protein hydrolysis (PH%) and peptide release (Section 2.2.5.2).

5.2.6 Microbial Proteome Profiling Analysis by LC-MS/MS

5.2.6.1 Protein Digestion and Purification

The cell pellet was resuspended in 200 µL of lysis buffer (50 mM Tris-HCl, 4 % sodium dodecyl sulfate, 10 mM dithiothreitol, pH 7.5) together with glass beads (0.2 g, 106 µm; Merck, Darmstadt, Germany). Cell lysis was achieved by using a FastPrep-24™ system (MP Biomedicals, Irvine, CA), in three rounds, each at 1600 rpm for 50 s. The suspension was then centrifuged at 13,000 × g for 5 min, and 30 µL of the supernatant was used for protein extraction. Protein concentration was

adjusted to 2 µg/µL after quantification with a NanoDrop© One UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 280 nm.

Suspension strapping was performed as described by Porcellato et al. (2024), with some modifications. Briefly, samples were incubated at 95 °C for 10 min using a SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific) and then cooled to room temperature. The samples were first treated with 3 µL of iodoacetamide (50 mM final concentration) for 20 min at room temperature in the dark, followed by acidification with 12.15 % v/v phosphoric acid. Subsequently, 160 µL of STrap buffer (90 % v/v methanol in 100 mM Tris-HCl, pH 7.1) was added to Pipette-tip columns (200 µL Eppendorf Research® plus G-3-pack, Eppendorf, Milan, Italy), pre-packed with dual-layer C18 resin (2215 - C18, Octadecyl, 47 mm, Empore, CDS, Oxford, PA, USA) and 12 layers of microquartz fiber paper (Art. No: 420050, grade MK 360, size 30 mm, Lot 3717, Ahlstrom Munksjö, Helsinki, Finland). The samples were then loaded onto the columns. After each reagent addition, the samples were centrifuged at 4,000 × g for 15 min, with the tip columns rotating 180° after each spin. Columns were washed with 50 µL of S-Trap buffer and centrifuged again. Proteins were then rehydrated with 100 µL of 50 mM ammonium bicarbonate and digested with trypsin (0.033 µg/µL in 50 mM ammonium bicarbonate, Merck) at 47 °C for 60 min. Digestion was stopped by sequentially adding 50 µL 0.5 % TFA and 100 µL 0.1 % TFA. Finally, peptides were eluted with 50 µL of 80 % acetonitrile in 0.1 % TFA and dried using a SVC-100H SpeedVac concentrator (Savant Control, Farmingdale, NY, USA) at 30 °C for 40 min. Dried peptides were stored at −20 °C until further analysis.

5.2.6.2 Label-free LC-MS/MS Proteomics

For Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) analysis, the dried suspension was resuspended in loading buffer (2 % acetonitrile, 0.05 % TFA), sonicated for 10 min in an ultrasonic water bath (Branson 2510, Soest, the Netherlands). The concentration was adjusted to 0.2 mg/mL using a NanoDrop© One UV-Vis spectrophotometer at 205 nm. Peptide analysis was conducted using a nanoElute ultra-high-performance liquid chromatography system (Bruker Daltonics, Bremen, Germany) coupled to a timsTOF Pro mass spectrometer (Bruker Daltonics), which integrates trapped ion mobility spectrometry with quadrupole time-of-flight (TIMS/QTOF) detection. Chromatographic separation was performed on a 25 cm × 75 µm PepSep Reprosil C18 reverse-phase column (1.5 µm particle size, 100 Å pore size), interfaced via a ZDV sprayer (Bruker Daltonics). The column temperature was maintained at 50 °C using an integrated column oven. Before sample injection, the column was equilibrated at 800 bar. Peptide separation was achieved at a constant flow rate of 300 nL/min using a multi-step gradient elution. Solvent B (99 % acetonitrile, 0.1 % formic acid) was ramped from 2 % to 25 % over 70 min, then increased to 37 % over 9 min, and further elevated to 95 % over the following 10 min. The final concentration was

maintained for an additional 10 min, resulting in a total run time of 99 min. Solvent A consisted of 0.1 % formic acid in water. The mass spectrometer operated in positive ion mode using the parallel accumulation-serial fragmentation method. Data acquisition was controlled using Compass Hystar (version 5.1.8.1) and timsControl (version 1.1.19.68). The mass range for MS acquisition spanned 100-1700 m/z. TIMS parameters included an inverse mobility ($1/K_0$) range of 0.85–1.4 V·s/cm², a ramp time of 100 ms, a ramp rate of 9.42 Hz, and full-duty-cycle operation (100 %). The capillary voltage was set to 1,400 V, with a dry gas flow of 3.0 L/min at 180 °C. For MS/MS acquisition, the system was configured to perform 10 PASEF ramps per cycle, with a total cycle time of 0.53 s. Precursor ion selection criteria included a charge state range of 0-5, a minimum intensity threshold of 2,500, and a target intensity of 20,000. Dynamic exclusion was set with a release time of 0.4 min, and collision-induced dissociation was applied using collision energies ranging from 27 to 45 eV.

5.2.6.3 Data Analysis

The raw LC-MS/MS data were processed using MaxQuant software version 2.0.3.1. Label-free quantification (LFQ) algorithm was performed with false discovery rates set to 0.01 at peptide and protein levels. Data were filtered to include at least one peptide per protein for protein identification. The peptide sequences were identified in MaxQuant using a custom-made protein database. The database was constructed by downloading all proteins of the genus *Lacticaseibacillus* from the proGenomes v3 database (https://progenomes.embl.de/, April 2025). Protein group LFQ intensities were filtered and normalized using random draws from a Gaussian distribution centered around a minimum value to control for missing proteins. Protein group LFQ intensities were further analyzed as described by Porcellato et al. (2024) using the DEP Bioconductor/R package (Zhang et al., 2018). The functional annotations of proteins in the database were obtained using the eggNOG 5.0 tool (Huerta-Cepas et al., 2019). The intensity of each protein group was then summed at the KEGG (Kyoto Encyclopedia of Genes and Genomes) ontology level (KO). The results were visualized using the basic R v 4.3.0 (The R Foundation for Statistical Computing, Vienna, Austria) packages ggplot2 and ggpubr (Villanueva & Chen, 2019).

5.2.7 Evaluation of the Biological Properties of the Fermentates

The fermentation supernatants from the stat phase were assessed for the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (as described in Section 3.2.4.1), the ferric reducing antioxidant power using the ferric reducing antioxidant power (FRAP) assay (Section 3.2.4.2), the angiotensin converting enzyme (ACE)-inhibitory activity (Section 3.2.4.3), and the antimicrobial activity (Section 3.2.4.4).

5.2.8 Statistical Analysis

All trials were carried out in triplicate, and values were expressed as the means ± standard deviation. Statistical analysis was performed using R v 4.3.0. Bartlett's test was used to assess homogeneity of variance; the difference between means was calculated using a t-test and a one-way analysis of variance (ANOVA), and the Tukey test was used to determine statistically significant differences among means (p<0.05).

5.3 Results and Discussion

5.3.1 Growth of *Lb. paracasei* AF43 in Different Media

In the present study, preliminary growth experiments were conducted to determine appropriate sampling points for subsequent analyses. For this purpose, turbidimetric growth curves of *Lb. paracasei* AF43 were determined in all tested media, namely whey protein concentrate with lactose (WM), pea protein concentrate with glucose (PM), and their mixtures at different ratios. Specifically, three WM:PM combinations were initially evaluated - 50:50 (WPM 50:50), 75:25 (WPM 75:25), and 25:75 (WPM 25:75) - to investigate the effect of mixed substrates on bacterial growth (Figure 5.1). The inclusion of these hybrid formulations was motivated by the growing interest in combining animal- and plant-derived protein sources to exploit their complementary nutritional and functional properties sustainably. The growth behavior of *Lb. paracasei* AF43 varied depending on the medium composition. The WPM 50:50 and WPM 25:75 formulations supported faster growth rates than WPM 75:25, suggesting that media containing higher proportions of PM (and thus more readily available glucose) promoted bacterial proliferation. Based on these results, WPM 50:50 was identified as the most effective mixed medium for supporting the growth of *Lb. paracasei* AF43 and was therefore selected, along with WM and PM, for subsequent investigation.

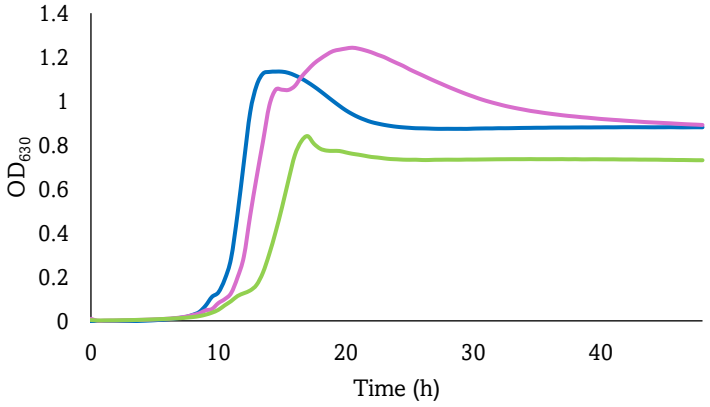

Figure 5.1 Kinetic growth curves of *Lb. paracasei* AF43 in WPM 50:50 (blue), WPM 75:25 (light green), and WPM 25:75 (purple)

Growth curves of the selected media were modeled using DMFit, enabling accurate determination of the times to reach the mid-exponential (mid-exp) and stationary (stat) growth phases. Table 5.1 summarizes the progression of fermentation in the different media. *Lb. paracasei* AF43 reached the mid-exp phase after 33 h in WM and after 11 h in both PM and WPM. The stat phase was achieved after 48 h in WM and after 14 h in PM and WPM, indicating that growth was markedly faster in the plant-based and hybrid media compared to WM.

Table 5.1 Fermentation progression of *Lb. paracasei* AF43 in pea medium (PM), whey medium (WM), and the mixed medium (WPM). Data are reported for each sampling point as mean ± standard deviation in terms of incubation time (h), cell viability (log cfu/mL), and pH

	PM			WM			WPM		
	Time	Viability	pH	Time	Viability	pH	Time	Viability	pH
start	0	$6.03^{cA} \pm 0.05$	$6.61^{aA} \pm 0.05$	0	$5.92^{cA} \pm 0.13$	$6.59^{aA} \pm 0.08$	0	$5.95^{cA} \pm 0.05$	$6.51^{aA} \pm 0.01$
mid-exp	11	$7.40^{bA} \pm 0.17$	$5.19^{bB} \pm 0.02$	33	$7.67^{bA} \pm 0.09$	$5.51^{bA} \pm 0.03$	11	$7.46^{bA} \pm 0.08$	$4.91^{bC} \pm 0.03$
stat	14	$7.94^{aA} \pm 0.12$	$4.40^{cC} \pm 0.03$	48	$7.99^{aA} \pm 0.21$	$4.83^{cA} \pm 0.02$	14	$8.17^{aA} \pm 0.18$	$4.56^{cB} \pm 0.01$

$^{a-c}$: means indicated with different letters differ significantly among time points within the same medium (p<0.05)
$^{A-C}$: means indicated with different letters differ significantly among media at the same time point (p<0.05)

Microbial enumeration revealed an initial bacterial concentration of approximately 6 log cfu/mL and an initial pH of 6.5-6.6 across all media. The bacterial load during the mid-exp and stat phases of growth didn't differ among the media, reaching approximately 7-8 log cfu/mL in the mid-exp phase and 8 log cfu/mL in the stat phase. Despite this, the trend of acidification differed slightly across the tested conditions. Rapid acidification occurred in WPM, with pH dropping below 5 at the mid-exp sampling time and to almost 4.5 at the stat phase. Acidification in PM was slightly slower, but it reached the lowest pH at the stat phase (4.40 ± 0.03). WM decreased the acidification rate of the strain, and higher pH values were obtained during fermentation: approximately 5.5 and 4.8 for the mid-exp and stat phases, respectively. Similarly, Wang et al. (2013) compared a dairy and a plant-based matrix (cow's milk and soy beverage) for the cultivation of *Lb. casei* Zhang. They observed slower milk growth, associated with delayed acidification and higher final pH values than in soy. This was explained by the higher buffering capacity of milk, which resists pH reduction, and by differences in nutrient availability: soy provides precursors that facilitate faster growth activation, whereas milk requires additional metabolic activity to access essential nutrients. However, differences in pH may also be influenced by variations in microbial metabolism, particularly in the types and amounts of organic acids produced during fermentation.

5.3.2 Proteome Overview: Focus on the Main Metabolisms

To investigate the different behavior of *Lb. paracasei* AF43 in WM, PM, and WPM media, label-free LC-MS/MS protein expression profiles were assessed from cells harvested during the mid-exponential and stationary growth phases. A total of 1,345 protein groups were detected across all

experimental conditions. A comparative analysis between the mid-exponential and stationary phases revealed minimal differences, with only one protein group differing between the two phases in WM and two protein groups differing between the two phases in WPM (data not shown). Given the slight differences, protein expression comparisons among the media were conducted using the data from the stationary phase. For clarity, in the following sections, unfermented WM, PM, and WPM refer to the non-inoculated media, whereas fermented WM, PM, and WPM indicate samples inoculated with *Lb. paracasei* AF43 and harvested at the stationary phase.

Considering the stationary phase, 1,006 protein groups were identified between the media. Overall, 111 protein groups showed statistically significant differences between at least two conditions. The HPLC-MS/MS signal was converted to LFQ intensity, which represents the relative protein abundance across samples (Van Den Bossche et al., 2025). Protein groups were then assigned to metabolic pathways using the KEGG KO annotation, and they were found to belong to 94 different pathways. The 30 pathways with higher LFQ intensity were associated with carbohydrate metabolism, ribosome metabolism, amino acid biosynthesis, fatty acid biosynthesis, and secondary metabolite biosynthesis. Among these, 23 pathways were statistically different between the conditions, primarily belonging to ribosome, carbohydrate, and protein metabolism.

5.3.2.1 Ribosome Metabolism

Ribosome metabolism serves as an indicator of a microorganism's metabolic activity. Under nutrient-rich and non-stressful conditions, cells allocate their proteome to ribosome production, enabling them to sustain rapid growth. Otherwise, when nutrients are limited and stress conditions prevail, the proteome allocation shifts toward stress response and survival, leading to downregulation of ribosome metabolism (Scott et al., 2014). In this study, ribosome metabolism was significantly lower in fermented WM (Figure 5.2), suggesting that *Lb. paracasei* AF43 may be stressed or required to adapt to nutrient sources of higher metabolic complexity, such as lactose, which, compared to glucose, requires an additional enzymatic step for utilization.

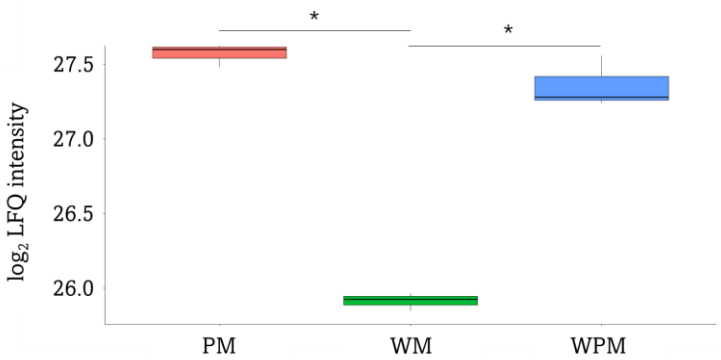

Figure 5.2 Log2 LFQ intensity of ribosome metabolic pathway in fermented pea medium (PM; red), whey medium (WM; green), and the mixed medium (WPM; blue) (p<0.05). Asterisks (*) indicate statistically significant differences between conditions (p<0.05)

In contrast, the same pathway was strongly upregulated in fermented PM and WPM, where favorable conditions, in particular high glucose availability, enable the proteome to shift toward rapid growth. These findings are consistent with previously observed growth kinetics.

5.3.2.2 Carbohydrate Metabolism

Lb. paracasei is usually able to metabolize a wide range of carbohydrates, unlike other species of lactobacilli with smaller genomes (e.g., *Lactobacillus delbrueckii* subsp. *bulgaricus*), which can utilize only a few sugars. Hence, this species adjusted its metabolic priorities in response to available nutrients (De Angelis et al., 2016). The data related to carbohydrate metabolism were first processed to generate an alluvial plot, which provides a clear visualization of how protein groups are distributed across different metabolic pathways. This approach highlights relative pathway abundances, enabling easy identification of the most enriched functional categories. As for carbohydrate metabolism, a total of 14 pathways were detected (Figure 5.3), among which glycolysis and gluconeogenesis, pyruvate metabolism, and galactose metabolism were the most enriched, as clearly shown by the flow distribution in the alluvial representation.

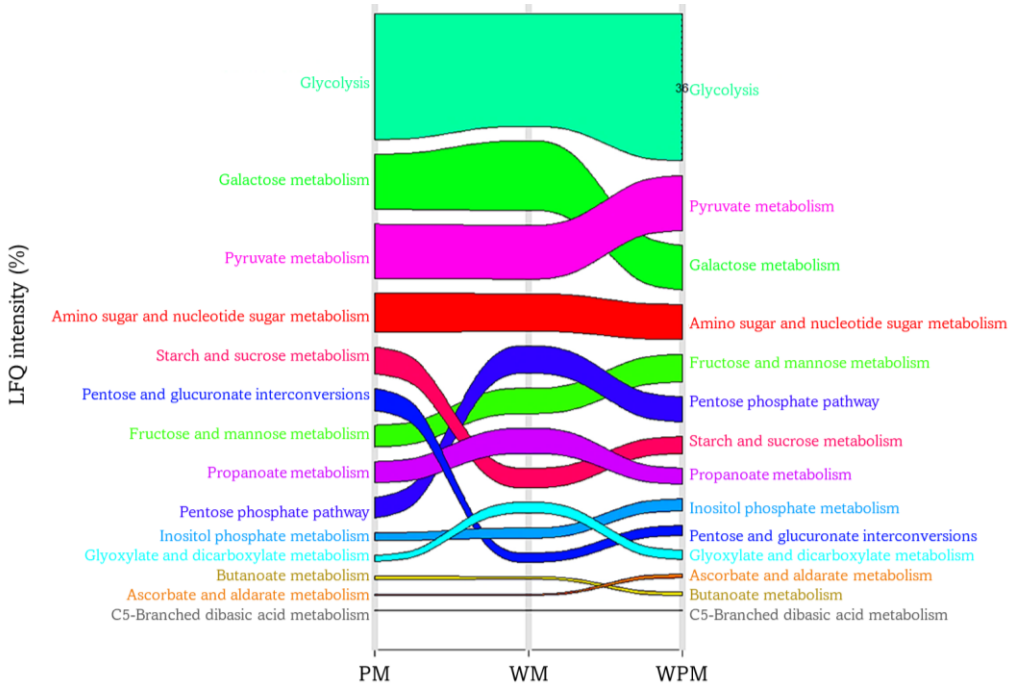

Figure 5.3 Alluvial plot of the 14 metabolic pathways belonging to carbohydrate metabolism detected in fermented pea medium (PM), whey medium (WM), and the mixed medium (WPM)

After providing a general overview of carbohydrate metabolism, the analysis focused on the main pathways *Lb. paracasei* uses for carbohydrate utilization, highlighting differences across conditions. Proteomic and metabolic profiling highlighted distinct carbohydrate utilization strategies of *L. paracasei* AF43 depending on the growth medium. In particular, Figure 5.4 illustrates the main carbohydrate utilization pathways in *Lb. paracasei* AF43 and shows the differential expression (p<0.05) of protein groups associated with carbohydrate metabolism when the strain was cultivated in PM,

WM, and WPM. The numerical values of the protein groups that were statistically different are also explicitly reported in Table S5.1.

Lb. paracasei is classified as a facultative heterofermentative species able to utilize both the homolactic and the hetero-lactic fermentation pathways depending on the available carbohydrate source and culture conditions. When fermenting hexoses such as glucose, the organism predominantly employs the Embden-Meyerhof-Parnas pathway, leading to pyruvate (Bintsis, 2018b). Unexpectedly, in PM, *Lb. paracasei* AF43 exhibited a clear shift toward heterofermentative metabolism despite the high glucose availability. This behavior was evidenced by the strong upregulation of several protein groups belonging to the phosphoketolase pathway, including glucose-6-phosphate dehydrogenase (Zwf), 6-phosphogluconolactonase (Pgl), 6-phosphogluconate dehydrogenase (Gnd), and xylulose-5-phosphate phosphoketolase (Xfp) (Figure 5.4; Table S5.1). Particularly, Xfp, considered a marker of heterofermentative LAB (Meile et al., 2001), was highly expressed, confirming the activation of this metabolism. This is characteristic of the phosphoketolase pathway in facultative heterofermentative LAB, which converts glucose into glyceraldehyde-3-phosphate (G3P). G3P is further converted to pyruvic acid and acetyl-phosphate, which can be converted into ethanol or acetic acid, together with CO_2 (Bintsis, 2018b).

The analytical data corroborate this metabolic shift. In unfermented PM, glucose was the only sugar detected (58.58 ± 0.13 g/L) (Table 5.2), and fermentation led to glucose depletion (54.88 ± 0.35 g/L). In parallel, the organic acid profile showed modest accumulation of lactic acid (0.16 ± 0.01 g/L), while acetic acid nearly doubled (0.46 ± 0.03 g/L). Genes associated with acetate formation from acetyl-phosphate (acetate kinase; AckA) were also upregulated. On the other hand, the G3P obtained was funneled into pyruvate formation through enzymatic steps common to the latter part of glycolysis. Accordingly, two key glycolytic enzymes - glyceraldehyde-3-phosphate dehydrogenase (Gap) and phosphoglycerate kinase (Pgk) - showed high expression levels in PM (Table S5.1), thereby sustaining NADH recycling and ATP generation via substrate-level phosphorylation (Ikemoto et al., 2003). Surprisingly, only a small fraction of the resulting pyruvate was converted to lactate via Ldh, as is commonly observed in LAB. Instead, citrate synthase (CS) was strongly upregulated in PM, redirecting pyruvate toward oxaloacetate and its condensation with acetyl-CoA to form citrate (De Angelis et al., 2016).

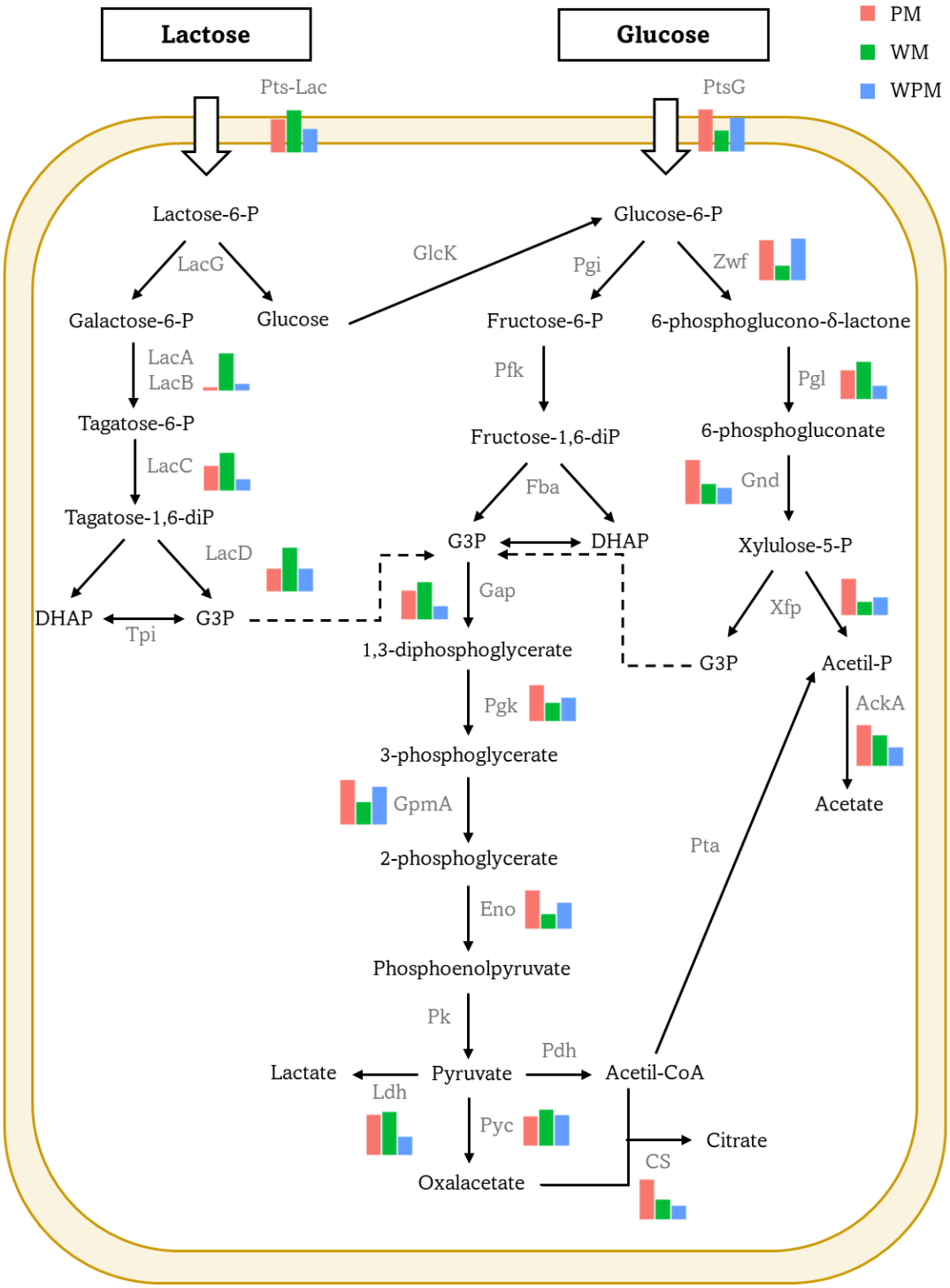

Figure 5.4 Carbohydrate metabolic pathways in *Lb. paracasei* AF43 cultivated in pea medium (PM), whey medium (WM), and the mixed medium (WPM). Protein groups identified by proteomics and associated with each enzymatic step are indicated in grey. Differential expression (log_2 LFQ intensity) of proteins with $p<0.05$ is represented by colored bars: red = PM, green = WM, blue = WPM. Abbreviations: GlcK, Glucokinase; Pgi, Glucose-6-phosphate isomerase; Pfk, Phosphofructokinase; Fba, Fructose-bisphosphate aldolase; Tpi, Triose-phosphate isomerase; Gap, Glyceraldehyde-3-phosphate dehydrogenase; Pgk, Phosphoglycerate kinase; GpmA, Phosphoglycerate mutase; Eno, Enolase; Pk, Pyruvate kinase; Ldh, Lactate dehydrogenase; Zwf, Glucose-6-phosphate dehydrogenase; Pgl, 6-phosphogluconolactonase; Gnd, 6-phosphogluconate dehydrogenase; Xfp, Xylulose-5-phosphate phosphoketolase; AckA, Acetate kinase; Pta, Phosphotransacetylase; Pyc, Pyruvate carboxylase; Pdh, Pyruvate dehydrogenase; CS, Citrate synthase; LacA and Lac B, Galactose-6-phosphate isomerase subunit A and B; LacC, Tagatose-6-phosphate kinase; LacD, Tagatose-1,6-bisphosphate aldolase; LacG, Phospho-β-galactosidase; PTS-Lac, Lactose-specific phosphotransferase system; PTS-G, Glucose-specific phosphotransferase system; P, Phosphate; DHAP, Dihydroxyacetone phosphate; G3P, Glyceraldehyde-3-phosphate

Table 5.2 Concentrations of sugars and organic acids (mg/mL; mean ± standard deviation) detected in the different media before and after growth of *Lb. paracasei* AF43 in pea medium (PM), whey medium (WM), and the mixed medium (WPM)

		Lactose	Glucose	Lactic acid	Acetic acid	Citric acid	Oxalic acid	Formic acid
PM	unfermented	n.d.	$58.58^{a} \pm 0.13$	n.d.	$0.24^{b} \pm 0.03$	$0.04^{b} \pm 0.002$	$0.23^{a} \pm 0.04$	$0.13^{a} \pm 0.003$
	fermented	n.d.	$54.88^{b} \pm 0.35$	$0.16^{a} \pm 0.01$	$0.46^{a} \pm 0.03$	$0.55^{a} \pm 0.01$	$0.13^{b} \pm 0.01$	n.d.
WM	unfermented	$58.60^{a} \pm 0.40$	n.d.	n.d.	$0.18^{a} \pm 0.01$	$0.02^{a} \pm 0.001$	$0.23^{b} \pm 0.003$	$0.15^{a} \pm 0.01$
	fermented	$55.20^{b} \pm 0.90$	n.d.	$0.53^{a} \pm 0.01$	$0.17^{a} \pm 0.003$	$0.02^{a} \pm 0.002$	$0.31^{a} \pm 0.01$	$0.15^{a} \pm 0.004$
WPM	unfermented	$27.01^{a} \pm 0.01$	$27.95^{a} \pm 0.01$	n.d.	$0.18^{b} \pm 0.01$	$0.03^{b} \pm 0.001$	$0.25^{a} \pm 0.02$	$0.16^{a} \pm 0.01$
	fermented	$27.51^{a} \pm 0.59$	$26.21^{b} \pm 0.57$	$0.6^{a} \pm 0.01$	$0.29^{a} \pm 0.04$	$0.08^{a} \pm 0.02$	$0.27^{a} \pm 0.02$	$0.16^{a} \pm 0.00$

Statistical difference ($p<0.05$) between unfermented and fermented media is indicated by different letters

n.d. denotes not detected

Accordingly, citric acid rose from 0.04 ± 0.002 to 0.55 ± 0.01 g/L after fermentation. The extracellular accumulation of citrate is unusual, as many LAB consume citrate via citrate permease and citrate lyase pathways rather than excreting it (Hugenholtz, 1993; Laëtitia et al., 2014). This finding may therefore reflect a strain-specific metabolic adaptation in PM. A likely explanation for this peculiar behavior is that the sharp pH decrease, combined with redox stress in PM, forced the strain to activate alternative metabolic routes. Indeed, acid and oxidative stresses are closely interconnected, as exposure to low pH not only perturbs the proton motive force but also promotes the accumulation of reactive oxygen species, thereby exacerbating oxidative damage and triggering antioxidant defense mechanisms (Feng & Wang, 2020; Papadimitriou et al., 2016). Furthermore, prior research on *Bifidobacterium longum* subjected to acid stress has shown that genes, including Xfp, Gap, AckA, and Eno, are upregulated to meet energy requirements under harsh pH conditions (Castro-López et al., 2023), which is consistent with our findings, as the same genes were also upregulated in PM. Supporting this hypothesis, proteins involved in glutathione metabolism were upregulated in PM (Figure S5.1). Glutathione, a low-molecular-weight antioxidant, protects cells by scavenging free radicals and maintaining intracellular redox homeostasis (Li et al., 2023). The enhanced activation of this pathway suggests that the acidic conditions reached during the stationary phase triggered oxidative stress, stimulating an increased antioxidant response, as observed in previous studies (Li et al., 2023; Lu & Holmgren, 2014).

In unfermented WM, where lactose was the sole available sugar (58.60 ± 0.40 g/L), the strain utilized lactose by first hydrolyzing it into glucose and galactose, with the resulting galactose being further metabolized through the tagatose-6-phosphate pathway, one of the main routes for galactose catabolism in LAB (Koskenniemi et al., 2009). The slower growth observed in fermented WM compared to PM and WPM is consistent with lactose utilization, since its catabolism requires more enzymatic steps than glucose (Bond et al., 1998). After being transported into the cell via the phosphotransferase system (PTS-Lac), lactose was initially hydrolyzed to glucose and galactose by β-galactosidase (LacG). In *Lb. paracasei* AF43, glucose was rapidly metabolized through the Embden-Meyerhof-Parnas pathway. In contrast, galactose was metabolized via the tagatose-6-phosphate pathway, generating intermediates that entered the latter part of glycolysis and were ultimately converted to pyruvate. Indeed, proteins of the lactose/galactose system were strongly upregulated in WM compared to the other two media, including PTS-Lac for lactose import, galactose-6-phosphate isomerase subunits A and B (LacA, LacB), tagatose-6-phosphate kinase (LacC), and tagatose-1,6-bisphosphate aldolase (LacD) (Figure 5.4; Table S5.1). This induction demonstrates that *Lb. paracasei* AF43 can efficiently hydrolyze lactose and utilize both glucose and galactose moieties. The sugar profile confirmed this: residual lactose decreased to 55.20 ± 0.90 g/L after fermentation, while glucose and galactose were undetectable, suggesting their rapid uptake and metabolism. Notably, this is a strain-dependent trait in *Lb. paracasei*, as some isolates are unable to metabolize

galactose and excrete it into the medium, which can lead to technological issues such as sugar accumulation or Maillard reactions (Wu et al., 2015). The strong expression of lactate dehydrogenase (Ldh) - the enzyme responsible for converting pyruvate into lactate - was supported by the detection of high amounts of lactic acid in the fermented WM (0.53 ± 0.01 g/L). By contrast, acetic acid levels remained stable, indicating a predominantly homolactic metabolism under these conditions.

In WPM, containing both glucose (27.95 ± 0.01 g/L) and lactose (27.01 ± 0.01 g/L), *Lb. paracasei* AF43 preferentially consumed glucose, as indicated by the upregulation of the glucose-specific phosphotransferase system (PtsG) and downregulation of PTS-Lac (Figure 5.4; Table S5.1). This pattern was confirmed by the sugar profile, showing glucose depletion (to 26.21 ± 0.57 g/L) while lactose remained nearly unchanged. Proteomic data indicated that homofermentative metabolism was predominant, supported by strong expression of Gap and Pgk. In WPM, the strain showed moderate activation of both Ldh, responsible for lactic acid production, and enzymes of the heterofermentative pathway. However, the high LFQ intensities of Ldh suggested that metabolism was predominantly directed toward lactic acid formation. Organic acid measurements supported this interpretation: lactic acid reached the highest concentration in WPM (0.60 ± 0.01 g/L), while acetic acid also increased moderately (from 0.18 ± 0.01 to 0.29 ± 0.04 g/L).

Oxalic acid and formic acid were present at low concentrations in both pea- and whey-based media, with only minor changes upon fermentation. In pea-based substrates, the presence of oxalic and formic acids is mainly attributable to their natural occurrence in legumes (Chai & Liebman, 2005). In whey-based substrates, both oxalic and formic acids likely reflect the bovine diet and intrinsic milk metabolism (Walstra et al., 2005).

The organic acid profiles corroborate the previously described acidification patterns (Table 5.2), with rapid acidification in WPM associated with high lactic acid accumulation and moderate acetic acid production. In PM, moderate lactic acid and higher levels of acetic and citric acids led to the sharpest drop in pH. In WM, limited acidification was associated with lactic acid production and stable levels of acetic and formic acid, consistent with the reduced pH decrease observed in this medium.

From a technological perspective, these findings have relevant implications for both product preservation and sensory quality. Acid production during fermentation contributes to a rapid decrease in pH, which may enhance microbial stability and extend product shelf-life by inhibiting spoilage and pathogenic bacteria. At the same time, the activation of heterofermentative pathways in PM and WPM, reflected by the accumulation of acetate, may positively influence the final product's sensory properties, contributing to flavor complexity (Kaleda et al., 2025). Finally, citrate accumulation in PM provides multiple benefits. One of the most relevant aspects is its role as a potent chelating agent, binding transition metals such as Fe^{2+} and Cu^{2+}. By limiting these pro-

oxidative reactions, citrate helps to preserve sensory quality, extend shelf life, maintain color and flavor stability in complex food systems, and - in the PM - may also have contributed to mitigating the oxidative stress experienced by the strain during growth (Durand et al., 2025; Mahoney Jr. & Graf, 1986). Citrate also contributes to nutritional quality, as citrate salts increase the solubility and gastrointestinal absorption of minerals such as calcium, magnesium, and iron, thereby enhancing their bioavailability compared to less soluble mineral salts (Schuchardt & Hahn, 2017).

5.3.2.3 Protein Metabolism

LAB rely mainly on their proteolytic system for amino acid supply, since they possess multiple amino acid auxotrophies (De Angelis et al., 2016). Moreover, in protein-based environments, they preferentially exploit their proteolytic system, which is a less energy-demanding strategy than *de novo* amino acid biosynthesis (Savijoki et al., 2006). The proteolytic system of LAB includes three key components: cell-envelope proteinases that break down extracellular proteins into oligopeptides (4-30 amino acids), a transport system that shuttles these peptides into the cell, and intracellular peptidases that convert them into amino acids or smaller peptides (2-3 amino acids) for metabolic use. Some of the peptides that aren't transported into the cell, along with the ones secreted during cell autolysis after being further hydrolyzed, can build up in the medium, and some of them may have bioactive properties. Additionally, proteolysis may lead to the accumulation of free amino acids in the medium, thereby enhancing protein digestibility and the nutritional value of the fermented product (Raveschot et al., 2018; Savijoki et al., 2006). Changing the fermentation medium can stimulate different proteases and transport mechanisms, promoting greater peptide release and accumulation in the medium, and enhancing the bioactive properties of the released peptides (Dineshbhai et al., 2022; Toldrá et al., 2018). In light of these considerations, the final part of this proteomic study focused on the expression of proteins associated with the proteolytic system of *Lb. paracasei* AF43 cultivated in PM, WM, and WPM.

A total of 61 protein groups referring to proteases and peptidases were detected, of which 17 are capable of hydrolyzing dietary proteins and thus potentially generating bioactive peptides (Table 5.3). PrtP is the main cell-envelope proteinase (CEP) of *Lb. paracasei,* which starts the proteolysis of dietary proteins outside the cell (Alcántara et al., 2016). This was the only protease identified, and no significant differences in its expression were detected across the media. However, the presence of PrtP in this strain represents a key metabolic advantage, as it enables the strain to access peptides and amino acids from complex protein substrates efficiently and also contributes to the release of peptides that may exert functional effects in the host or influence the sensory properties of the fermented product. Some lactobacilli, including *Lb. paracasei*, produce little to no CEP and often depend on other starter cultures for oligopeptide production. However, they compensate by highly expressing peptide transporters and intracellular peptidases (Savijoki et al., 2006).

Consistently, 16 peptidases were detected in the proteome of *Lb. paracasei* AF43, comprising 4 endopeptidases (PepF, PepF2, PepO, and PepE), 4 aminopeptidases (PepN, PepC, PepS, and Map), a carboxypeptidase (Pcp), 4 proline-specific peptidases (PepQ, PepR, PepX, and PepP), and 3 di-peptidases (PepD2, PepV, and PepDA). Among these, PepO, PepE, and PepN showed statistically significant differences in expression between the tested conditions (Table 5.3).

Table 5.3 List of peptidases and proteases and oligopeptide permease system (Opp) transporters expressed by *Lb. paracasei* AF43 in fermented pea medium (PM), whey medium (WM), and the mixed medium (WPM). Data are expressed as mean ± standard deviation

Name	EC number	Intensity (log_2 LFQ)			Function
		PM	WM	WPM	
Peptidases and proteases					
PrtP	3.4.21.96	$19.79^{a} \pm 0.27$	$19.24^{a} \pm 0.15$	$18.86^{a} \pm 0.68$	Serine protease
PepF	-	$17.55^{a} \pm 1.62$	$16.68^{a} \pm 1.04$	$17.82^{a} \pm 0.47$	Endopeptidase
PepF2	-	$17.61^{a} \pm 0.30$	$17.76^{a} \pm 0.62$	$19.34^{a} \pm 0.41$	Endopeptidase
PepE	3.4.22.40	$17.88^{ab} \pm 1.18$	$15.75^{b} \pm 0.34$	$19.20^{a} \pm 0.63$	Endopeptidase
PepO	-	$19.46^{ab} \pm 0.57$	$17.43^{b} \pm 1.07$	$20.22^{a} \pm 0.49$	Endopeptidase
PepN	-	$18.04^{ab} \pm 0.39$	$17.45^{b} \pm 0.34$	$19.81^{a} \pm 0.44$	Aminopeptidase
PepC	3.4.22.40	$20.39^{a} \pm 0.44$	$19.95^{a} \pm 0.25$	$21.07^{a} \pm 0.52$	Aminopeptidase
PepS	-	$17.94^{a} \pm 1.19$	$16.14^{a} \pm 0.22$	$16.97^{a} \pm 1.90$	Aminopeptidase
Map	3.4.11.18	$17.22^{a} \pm 1.41$	$18.29^{a} \pm 0.35$	$18.70^{a} \pm 0.92$	Aminopeptidase
Pcp	3.4.19.3	$17.63^{a} \pm 0.46$	$17.86^{a} \pm 0.68$	$18.08^{a} \pm 0.69$	Carboxypeptidase
PepQ	3.4.13.9	$20.13^{a} \pm 1.91$	$18.47^{a} \pm 1.00$	$17.60^{a} \pm 1.03$	Proline-specific peptidase
PepR	3.4.11.5	$17.96^{a} \pm 0.84$	$16.69^{a} \pm 1.45$	$18.35^{a} \pm 0.48$	Proline-specific peptidase
PepX	3.4.14.11	$15.53^{a} \pm 0.61$	$16.10^{a} \pm 0.47$	$15.48^{a} \pm 0.83$	Proline-specific peptidase
PepP	3.4.11.9	$18.53^{a} \pm 1.12$	$16.27^{a} \pm 1.12$	$17.29^{a} \pm 0.87$	Proline-specific peptidase
PepD2	-	$16.46^{a} \pm 0.28$	$17.34^{a} \pm 0.38$	$17.43^{a} \pm 1.66$	Dipeptidase
PepV	3.5.1.18	$18.08^{a} \pm 0.84$	$19.55^{a} \pm 0.22$	$17.85^{a} \pm 1.34$	Dipeptidase
PepDA	-	$17.40^{a} \pm 1.54$	$16.15^{a} \pm 0.32$	$17.72^{a} \pm 0.97$	Dipeptidase
Opp transporters					
OppD	-	$20.32^{a} \pm 0.23$	$20.23^{a} \pm 0.10$	$19.69^{a} \pm 1.32$	ATP-binding
OppF	-	$17.11^{a} \pm 0.84$	$16.18^{a} \pm 1.26$	$18.11^{a} \pm 0.68$	ATP-binding
OppB	-	$18.67^{a} \pm 0.63$	$17.62^{a} \pm 1.02$	$17.36^{a} \pm 0.32$	Membrane channel
OppA	-	$20.09^{a} \pm 0.25$	$19.38^{a} \pm 0.11$	$20.47^{a} \pm 0.71$	Peptide binding

Statistical difference ($p<0.05$) within each media is indicated by different letters

In particular, they were downregulated in fermented WM and upregulated in WPM, suggesting higher peptidase activity in WPM, followed by an intermediate level in PM. The modulation of

peptidase expression may not only affect the strain's amino acid supply but also the release of peptides with distinct sequences, as each peptidase has specific substrate preferences. PepN mainly hydrolyzes peptides (4 to 6 amino acids) containing lysine and leucine, or hydrophobic amino acids (Zou et al., 2024). PepO is active on oligopeptides with a length of 5 to 30 amino acids and containing arginine and methionine residues (Rodríguez-Serrano et al., 2018). PepO cleaves bonds within the peptide chain, without specificity (Griffiths & Tellez, 2013). Since peptides' biological activities depend on their amino acid composition, this could influence the FF's health-promoting potential. In line with this, Chen et al. (2024) showed that in goat milk fermentation, differential expression of proteolytic enzymes by LAB was associated with the generation of peptides with ACE-inhibitory activity, highlighting the functional relevance of the proteolytic system.
The oligopeptide permease system (Opp) plays a key role in transporting oligopeptides generated by CEP cleavage into the cytoplasm, where they can be further hydrolyzed by intracellular peptidases (Rodríguez-Serrano et al., 2018). All core components of the Opp system, including OppA, OppD, OppB, and OppF, were equally detected in the proteome of *Lb. paracasei* AF43 under all tested conditions (Table 5.3). By summing the LFQ intensity values ($\log_2$ LFQ intensity) for the detected proteases, peptidases, and Opp transporters, it was possible to obtain their cumulative expression levels across the different media. As shown in Figure 5.5, the cumulative expression of peptidases and proteases and Opp transporters was significantly higher in fermented WPM and PM compared to fermented WM (p<0.05), suggesting high proteolytic activity and peptide uptake in these media, probably to support rapid growth and high amino acid needs.

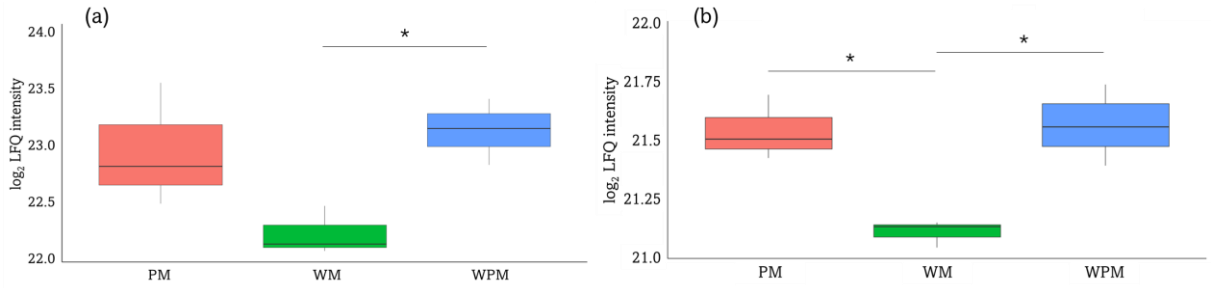

Figure 5.5 Cumulative expression levels of (a) peptidases and proteases, and (b) Opp transporters in *Lb. paracasei* AF43 cultured in pea medium (PM), whey medium (WM), and the mixed medium (WPM). Expression is represented as the sum of $\log_2$ LFQ intensities of all protein groups assigned to each functional category. Asterisks (*) indicate statistically significant differences between conditions (p<0.05)

These findings are consistent with the analytical data of protein hydrolysis (Table 5.4). Fermented PM and WPM exhibited the highest PH% (84.97 ± 1.89 % and 30.06 ± 0.72 %, respectively), whereas fermented WM showed only moderate hydrolysis (24.79 ± 2.07 %). However, this higher proteolysis in PM and WPM did not translate into peptide accumulation, since relatively low levels of small peptides were observed in both conditions. In contrast, WM accumulated the most

significant amount of low-molecular-weight peptides despite its limited proteolytic activity, reaching 0.81 ± 0.08 mg/mL in the 6-3 kDa range and 0.51 ± 0.08 mg/mL in the <3 kDa fraction.

Table 5.4 Extent of protein hydrolysis (PH%) and concentration of peptides (mg/mL) in the 6–3 kDa and <3 kDa MW ranges released after fermentation of pea medium (PM), whey medium (WM), and the mixed medium (WPM) by *Lb. paracasei* AF43. Data are expressed as mean ± standard deviation

	PH (%)	Peptide 6-3 kDa (mg/mL)	Peptide <3 kDa (mg/mL)
PM	84.97^{a} ± 1.89	0.09^{c} ± 0.01	0.24^{b} ± 0.04
WM	24.79^{c} ± 2.07	0.81^{a} ± 0.08	0.51^{a} ± 0.08
WPM	30.06^{b} ± 0.72	0.66^{b} ± 0.01	0.24^{b} ± 0.02

Statistical difference (p<0.05) within each column is indicated by different letters

Together, these results suggest that, in fermented PM and WPM, higher peptidase expression promoted extensive protein hydrolysis and peptide degradation, thereby limiting the accumulation of small peptides in the extracellular environment. Overall, these findings highlight how the fermentation medium modulates the proteolytic activity of *Lb. paracasei* AF43 and shapes the resulting peptide profile, with potential implications for the nutritional and functional properties of the fermented product. The amino acid composition before and after fermentation of the different media was also evaluated, as proteolytic activity may influence free amino acid availability and indicate their selective consumption or release. Table 5.5 shows the relative abundance (%) of amino acids detected in PM, WM, and WPM before and after growth of *Lb. paracasei* AF43. In PM, *Lb. paracasei* AF43 consumed most of the amino acids originally present in the unfermented medium. It did not release significant amounts through proteolysis, reflecting its strong requirement for amino acids to sustain growth.

Interestingly, proline, which was absent in the unfermented medium, appeared at high concentrations after fermentation. This likely reflects the proteolysis of proline-rich regions of pea proteins. Although proline is a non-essential amino acid, it plays an important role in peptide structure and is frequently enriched in bioactive peptides, including those with ACE-inhibitory activity (Chen et al., 2024). In WM, *Lb. paracasei* AF43 modulated the amino acid profile in a more balanced way, leading to the depletion of some amino acids, such as tyrosine and leucine, while maintaining others unchanged and increasing the content of proline and lysine. Proteolysis was less extensive, in accordance with previous results, but selective amino acid metabolism led to the accumulation of specific residues. This again reflects the strain's ability to hydrolyze proline-rich regions, also found in whey proteins. Notably, the accumulation of lysine could enhance the nutritional value of the fermented product, since lysine is an essential amino acid. Lastly, in WPM, the strain consumed threonine and tyrosine while releasing a small amount of methionine. Once again, fermentation led to a significant accumulation of proline.

Table 5.5 Relative abundance (%; mean ± standard deviation) of amino acids detected in pea medium (PM), whey medium (WM), and the mixed medium (WPM) before and after growth of *Lb. paracasei* AF43

		Histidine	Aspartic acid	Threonine	Cystine	Proline	Tyrosine	Methionine	Valine	Leucine	Cysteine	Lysine
PM	unfermented	$34.65^{a} \pm 11.2$	n.d.	$14.67^{a} \pm 4.03$	$11.15^{a} \pm 3.22$	n.d.	$1.12^{a} \pm 0.34$	$4.21^{a} \pm 1.42$	$0.42^{a} \pm 0.16$	$33.26^{a} \pm 9.58$	n.d.	n.d.
	fermented	$15.86^{b} \pm 4.59$	n.d.	$9.49^{a} \pm 6.64$	$8.04^{a} \pm 3.45$	$64.02^{a} \pm 13.97$	$0.26^{b} \pm 0.23$	$0.73^{b} \pm 0.94$	$0.28^{b} \pm 0.17$	$1.27^{b} \pm 0.11$	n.d.	n.d.
WM	unfermented	n.d.	n.d.	$9.8^{a} \pm 2.46$	$4.33^{a} \pm 1.92$	$26.86^{b} \pm 6.25$	$45.4^{a} \pm 9.07$	n.d.	n.d.	$3.12^{a} \pm 0.19$	$1.44^{a} \pm 0.28$	$9.44^{b} \pm 2.54$
	fermented	n.d.	$1.91^{a} \pm 0.56$	$5.26^{a} \pm 0.41$	$4.29^{a} \pm 0.44$	$41.64^{a} \pm 4.24$	$23.83^{b} \pm 4.85$	n.d.	n.d.	$0.57^{b} \pm 0.07$	$2.63^{a} \pm 0.69$	$22.7^{a} \pm 6.73$
WPM	unfermented	$13.28^{a} \pm 2.77$	n.d.	$13.63^{a} \pm 0.42$	$10.37^{a} \pm 0.07$	$49.66^{b} \pm 0.49$	$11.61^{a} \pm 1.17$	n.d.	n.d.	$1.45^{a} \pm 0.05$	n.d.	n.d.
	fermented	$15.16^{a} \pm 0.59$	n.d.	$10.54^{b} \pm 1.17$	$8.98^{a} \pm 1.16$	$62.00^{a} \pm 29.06$	n.d.	$1.57^{a} \pm 0.25$	n.d.	$1.75^{a} \pm 0.55$	n.d.	n.d.

Statistical difference ($p<0.05$) between unfermented and fermented media is indicated by different letters

n.d. denotes not detected

This trend highlights the preferential release of proline across different media, whereas other amino acids may be selectively utilized or released only partially, depending on the media.

5.3.3 Biological Properties of the Fermentates

Proteomics and analytical data laid the foundation for further exploration of the biological functions of microbial protein hydrolysates obtained from the fermentation of PM, WM, and WPM.

5.3.3.1 Antioxidant Activity

As a first step, antioxidant activity was assessed using both the FRAP and DPPH assays (Figure 5.6), as multiple methods are essential to capture different mechanisms of action. Indeed, while the DPPH assay measures the ability of compounds to donate hydrogen atoms or electrons to neutralize free radicals (Cui et al., 2022), the FRAP assay evaluates the reducing power of antioxidants by their capacity to reduce Fe^{3+} to Fe^{2+} (Benzie & Strain, 1996).

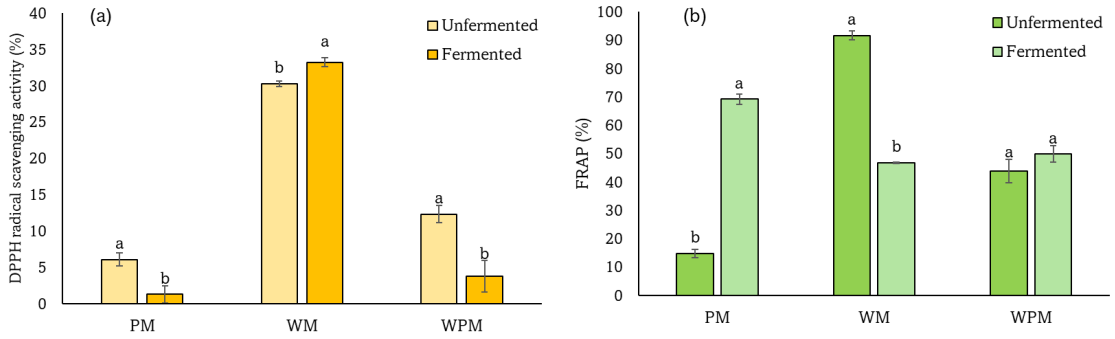

Figure 5.6 Antioxidant activity (%; mean ± standard deviation) measured with DPPH (a) and FRAP (b) assays before and after growth of *Lb. paracasei* AF43 in pea medium (PM), whey medium (WM), and the mixed medium (WPM). Statistical difference (p<0.05) between unfermented and fermented media is indicated by different letters

Both whey and pea proteins may exhibit antioxidant activity (Di Filippo et al., 2024; Pownall et al., 2010); therefore, the non-fermented substrates were initially analyzed as reference points. Unfermented PM exhibited a moderate intrinsic antioxidant capacity, with FRAP values of 14.80 ± 1.49 % and DPPH values of 6.11 ± 0.94 %. Both unfermented WM and WPM showed higher basal antioxidant activities: WM stood out for its strong reducing power (91.69 ± 1.48 %) and high radical scavenging activity (30.29 ± 0.4 %), while WPM displayed intermediate values (43.85 ± 4.08 %; DPPH 12.34 ± 1.22 %).

Overall, fermentation differentially modulated antioxidant potential depending on the protein substrate. In PM, fermentation increased reducing power, with FRAP rising to 69.17 ± 1.82 %, while DPPH activity decreased. This shift highlights a mechanism dominated by Fe^{3+} reduction rather than radical scavenging. Interestingly, although fermentation of PM was associated with lower overall peptide release than WM and WPM, it still showed a remarkable increase in FRAP activity. This effect may not be related to peptide accumulation but rather to citric acid accumulation, which

can strongly contribute to reducing power through its metal-chelating and redox-modulating properties, as previously described (Durand et al., 2025; Mahoney Jr. & Graf, 1986).

In WM, fermentation markedly reduced FRAP to 46.79 ± 0.24 %, but enhanced DPPH activity to 33.23 ± 0.60 %. This indicates that fermentation by *Lb. paracasei* AF43 preserved antioxidant potential but redirected it toward radical scavenging, possibly via the release of amino acids and peptides possessing this capacity. Indeed, fermented WM showed a higher concentration of peptides, and the enrichment in lysine in fermented WM is particularly relevant (Table 5.5), since lysine can directly participate in radical scavenging or act as a precursor for antioxidant peptides (Akbarian et al., 2022; Olin-Sandoval et al., 2019). The structural features of these peptides, including hydrophobicity and the presence of basic residues, are known to play a decisive role in antioxidant potential (Ren et al., 2008). For WPM, fermentation didn't impact FRAP, while reducing DPPH activity to 3.80 ± 2.14 %.

5.3.3.2 ACE-inhibitory Activity

In parallel, the ability of the hydrolysates to inhibit ACE was evaluated (Figure 5.7), as this mechanism plays a crucial role in blood pressure regulation by catalyzing the conversion of angiotensin I into the vasoconstrictor angiotensin II. By inhibiting the enzyme, this conversion is blocked, producing a hypotensive effect (Zheng et al., 2022). ACE-inhibitory activity was determined by HPLC, monitoring the consumption of the synthetic substrate hippuryl-histidyl-leucine and the concomitant formation of the product hippuric acid. From the HPLC peak areas, the percentage of ACE inhibition was calculated.

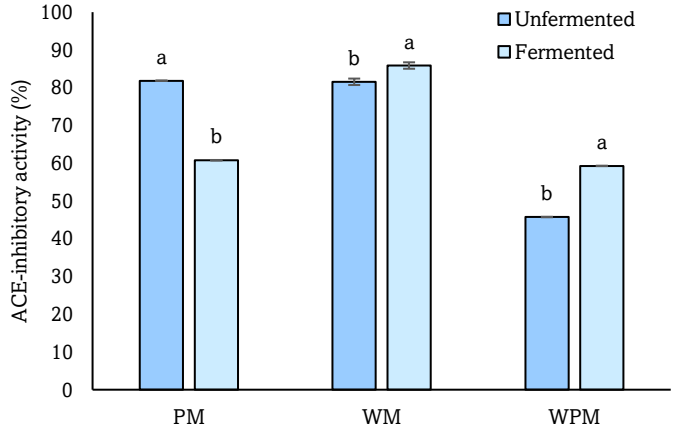

Figure 5.7 ACE-inhibitory activity (%; mean ± standard deviation) before and after growth of *Lb. paracasei* AF43 in pea medium (PM), whey medium (WM), and the mixed medium (WPM). Statistical difference (p<0.05) between unfermented and fermented media is indicated by different letters

Fermentation influenced the ACE-inhibitory activity of the different protein matrices in distinct ways. In PM, activity decreased (p<0.05) to 60.78 ± 0.85 %, indicating that although pea proteins displayed strong intrinsic inhibitory capacity, fermentation by *Lb. paracasei* AF43 reduced their effectiveness. In contrast, both WM and WPM showed enhanced ACE-inhibitory activity upon

fermentation (p<0.05). In fermented WM, ACE-inhibitory activity increased by approximately 4 %, whereas in WPM it rose significantly from 45.74 ± 0.24 % to 59.28 ± 0.68 %.

Peptide quantification by SE-HPLC analysis supports these outcomes (Table 5.4): WM showed the highest peptide release, particularly in the <3 kDa range, which is often associated with bioactivity. WPM followed WM in terms of peptide release, and both matrices accumulated significant amounts of proline (Table 5.5), suggesting that *Lb. paracasei* AF43 metabolism was directed toward protein fractions enriched in proline. Proline is known to play a key role in peptide sequences, supporting ACE-inhibitory activity (Chen et al., 2024), thereby strengthening the mechanistic link between peptide composition and bioactivity.

Interestingly, WPM showed the greatest relative enhancement in ACE-inhibitory activity, suggesting that the synergistic effects of both protein sources are evident when used together as fermentation substrates. The combined effect may arise from complementary peptide pools derived from whey and pea proteins, thereby enhancing the diversity of bioactive sequences and increasing inhibitory potential.

5.3.3.3 Antimicrobial Activity

Microbial protein fermentates exhibit antimicrobial activity against a wide range of bacteria, including antibiotic-resistant strains, thereby contributing to infection control and food safety (Hernández Figueroa et al., 2024). The antimicrobial activity of PM, WM, and WPM after fermentation with *Lb. paracasei* AF43 was evaluated against six food-relevant pathogens and spoilage bacteria: three Gram-positive (*Enterococcus* spp., *Listeria monocytogenes*, *Staphylococcus aureus*) and three Gram-negative species (*Escherichia coli*, *Salmonella enterica*, *Pseudomonas fluorescens*), with strains pooled within each genus to account for natural variability. Antimicrobial effects were assessed using a turbidimetric approach, in which inhibitory compounds can alter microbial growth by prolonging the lag phase, reducing the maximum growth rate, or lowering the final cell density (Innocente et al., 2023). Growth kinetics were modeled using the Gompertz equation to determine the lag time (λ, h), the maximum specific growth rate (μ_{max}, log OD/h), and the growth amplitude (A, OD).

Firstly, baseline growth in unfermented whey and pea protein media was compared to BHI as a control to evaluate the intrinsic effects of these substrates (Table 5.6). Overall, the unfermented PM and WPM media had a modest impact on the target strains, with growth kinetics largely comparable to those in BHI. In contrast, unfermented WM reduced lag time and increased μ_{max} in several cases, indicating that whey can stimulate the early stages of bacterial proliferation. However, despite this accelerated onset, the final optical density often remained equal to or lower than that observed in BHI, not enhancing the overall biomass accumulation. Gram-positive bacteria exhibited shorter lag times and higher growth rates in the presence of unfermented WM, whereas

WPM occasionally led to increased A values. Among Gram-negative species, *Salm. enterica* showed extended lag times and reduced A in all media, suggesting a potential inhibitory effect, while *E. coli* and *Ps. fluorescens* were minimally affected.

Table 5.6 Effect of the unfermented pea medium (PM), whey medium (WM), and mixed medium (WPM) on growth parameters of *L. monocytogenes*, *Staph. aureus*, *Enterococcus* spp., *E. coli*, *Salm. enterica*, and *Ps. fluorescens*. Data are expressed as mean ± standard deviation

	λ (h)	μ_{max} (log OD/h)	A (OD)
L. monocytogenes			
BHI	7.21 ± 0.13	0.31 ± 0.02	1.08 ± 0.03
PM	7.06 ± 0.05	0.31 ± 0.01	1.21 ± 0.12
WM	3.48* ± 0.03	0.49* ± 0.02	1.22* ± 0.03
WPM	6.65 ± 0.26	0.25 ± 0.03	1.27* ± 0.05
Staph. aureus			
BHI	5.69 ± 0.4	0.65 ± 0.13	1.49 ± 0.12
PM	5.06 ± 0.05	0.41* ± 0.01	1.27 ± 0.04
WM	3.43* ± 0.04	0.41* ± 0.01	1.18* ± 0.03
WPM	4.72 ± 0.03	0.61 ± 0.04	1.59 ± 0.01
Enterococcus spp.			
BHI	4.89 ± 0.01	0.89 ± 0.01	1.42 ± 0.02
PM	5.14 ± 0.09	0.79 ± 0.01	1.48 ± 0.03
WM	3.72* ± 0.04	0.62* ± 0.02	1.21* ± 0.01
WPM	4.31* ± 0.06	0.59* ± 0.02	1.57* ± 0.02
E. coli			
BHI	2.41 ± 0.13	0.39 ± 0.03	1.49 ± 0.02
PM	2.85 ± 0.07	0.44 ± 0.02	1.41* ± 0.01
WM	2.69 ± 0.02	0.37 ± 0.02	1.31* ± 0.02
WPM	3.04 ± 0.15	0.43 ± 0.02	1.58* ± 0.00
Salm. enterica			
BHI	2.88 ± 0.03	0.23 ± 0.04	1.22 ± 0.03
PM	4.18* ± 0.07	0.79* ± 0.01	1.06* ± 0.06
WM	3.51* ± 0.02	0.42* ± 0.03	1.16 ± 0.05
WPM	4.15* ± 0.07	0.73* ± 0.03	1.06* ± 0.02
Ps. fluorescens			
BHI	4.69 ± 0.01	0.27 ± 0.03	1.57 ± 0.02
PM	5.3* ± 0.01	0.32 ± 0.08	1.39* ± 0.01
WM	4.68 ± 0.02	0.31 ± 0.02	1.28* ± 0.01
WPM	4.73 ± 0.23	0.36 ± 0.06	1.59 ± 0.01

μ_{max} is the maximum specific growth rate; λ is the lag time; A is the upper asymptote
Statistical differences (p<0.05) from the control (BHI) are indicated by an asterisk (*)

The effect of fermentation depended on both the protein matrix used and the specific microbial target. Figure 5.8 illustrates, as an example, the turbidimetric growth curves of *L. monocytogenes* in

the presence of a fermentate, compared with those in the corresponding unfermented media across different protein substrates. As shown, fermented PM and WPM generally had little to no effect on *L. monocytogenes* growth compared to unfermented media, as their lag phases, μ_{max}, and A were similar. In contrast, the fermented WM strongly inhibited bacterial growth, resulting in a prolonged lag phase and a lower final concentration than with unfermented WM.

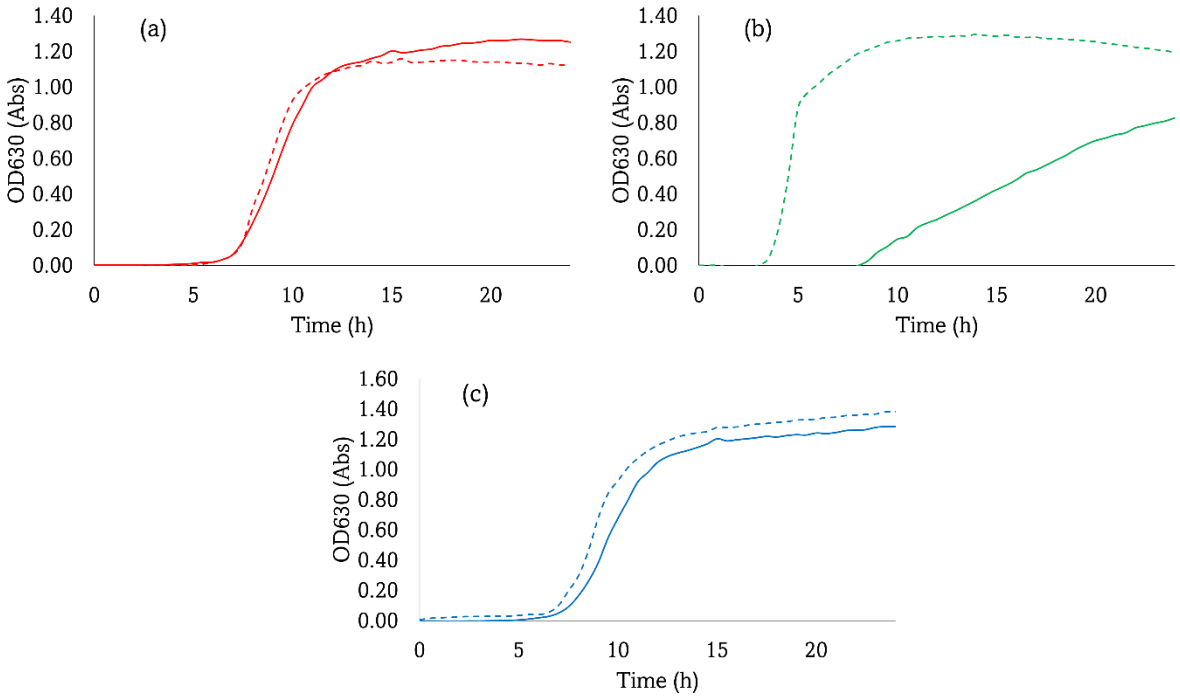

Figure 5.8 Turbidimetric growth curves of *L. monocytogenes* in the presence of a fermentate (continuous line) or unfermented media (dotted line). (a) Red: pea medium (PM), (b) Green: whey medium (WM), (c) Blue: mixed medium (WPM)

As shown in Table 5.7, this trend was also observed for other Gram-positive targets: fermented PM and WPM had minimal impact on the growth of *Staph. aureus* and *Enterococcus* spp., whereas fermented WM significantly extended the lag phase and reduced the μ_{max} of these pathogens. However, *L. monocytogenes* was the most affected target. This is particularly noteworthy considering its inherent resistance to stressful conditions and its high incidence and lethality, which pose a substantial risk to immunocompromised populations (Innocente et al., 2023). In contrast, Gram-negative bacteria were largely unaffected by any of the fermentates (Table 5.8).

Several factors may have contributed to the pronounced inhibitory activity against Gram-positive bacteria observed in fermented WM. The culture medium maintained a pH of approximately 6.6 following the addition of the fermentate, and the abundance of organic acids, particularly in fermented PM and WPM, did not translate into more potent inhibition, indicating that acidity was not a decisive factor.

Table 5.7 Effect of the fermentates on growth parameters of Gram-positive microbial targets. Data are expressed as mean ± standard deviation

Sample		*L. monocytogenes*			*Staph. aureus*			*Enterococcus* spp.		
		λ	μ_{max}	Amplitude	λ	μ_{max}	Amplitude	λ	μ_{max}	Amplitude
PM	unfermented	7.06 ± 0.05	0.31 ± 0.01	1.21 ± 0.12	5.06 ± 0.15	0.41 ± 0.01	1.27 ± 0.04	5.14 ± 0.09	0.79 ± 0.01	1.48 ± 0.03
	fermented	6.83 ± 0.27	0.24* ± 0.01	1.22 ± 0.02	3.95 ± 0.12	0.45 ± 0.03	1.56* ± 0.03	4.47* ± 0.06	0.58* ± 0.05	1.53 ± 0.03
WM	unfermented	3.48 ± 0.03	0.49 ± 0.02	1.22 ± 0.03	3.43 ± 0.04	0.41 ± 0.01	1.18 ± 0.03	3.72 ± 0.04	0.62 ± 0.02	1.21 ± 0.01
	fermented	7.7* ± 0.04	0.06* ± 0.01	0.78* ± 0.06	5.4* ± 0.11	0.23* ± 0.01	1.26* ± 0.01	4.15* ± 0.15	0.27* ± 0.04	1.16 ± 0.03
WPM	unfermented	6.65 ± 0.26	0.25 ± 0.03	1.27 ± 0.05	4.72 ± 0.03	0.61 ± 0.04	1.59 ± 0.01	4.31 ± 0.06	0.59 ± 0.02	1.57 ± 0.02
	fermented	6.91 ± 0.61	0.22 ± 0.03	1.22 ± 0.00	4.61* ± 0.02	0.66 ± 0.02	1.52* ± 0.02	4.46 ± 0.09	0.59 ± 0.09	1.53* ± 0.01

[1] μ_{max} is the maximum specific growth rate; λ is the lag time; A is the upper asymptote
[2] Statistical differences (p<0.05) between fermented and unfermented medium are indicated by an asterisk (*)

Table 5.8 Effect of the fermentates on growth parameters of Gram-negative microbial targets. Data are expressed as mean ± standard deviation

Sample		*E. coli*			*Salm. enterica*			*Ps. fluorescens*		
		λ	μ_{max}	Amplitude	λ	μ_{max}	Amplitude	λ	μ_{max}	Amplitude
PM	unfermented	2.85 ± 0.07	0.44 ± 0.02	1.41 ± 0.01	4.18 ± 0.07	0.79 ± 0.02	1.06 ± 0.06	5.3 ± 0.01	0.32 ± 0.08	1.39 ± 0.01
	fermented	3.17* ± 0.05	0.47 ± 0.01	1.60* ± 0.01	4.31* ± 0.01	0.82 ± 0.01	1.08 ± 0.02	4.71 ± 0.8	0.31 ± 0.01	1.56* ± 0.01
WM	unfermented	2.69 ± 0.02	0.37 ± 0.02	1.31 ± 0.02	3.51 ± 0.02	0.42 ± 0.03	1.16 ± 0.05	4.68 ± 0.02	0.31 ± 0.02	1.28 ± 0.01
	fermented	1.4* ± 0.32	0.2* ± 0.01	1.38 ± 0.04	1.7* ± 0.18	0.12* ± 0.01	1.31* ± 0.06	4.75 ± 0.33	0.18* ± 0.01	1.44* ± 0.05
WPM	unfermented	3.04 ± 0.15	0.43 ± 0.02	1.58 ± 0.00	4.15 ± 0.07	0.73 ± 0.03	1.06 ± 0.02	4.73 ± 0.23	0.36 ± 0.06	1.59 ± 0.01
	fermented	3.13 ± 0.06	0.44 ± 0.01	1.59 ± 0.01	4.42* ± 0.05	0.87* ± 0.03	1.09 ± 0.05	4.65 ± 0.25	0.25 ± 0.02	1.63* ± 0.01

μ_{max} is the maximum specific growth rate; λ is the lag time; A is the upper asymptote

Statistical differences (p<0.05) between fermented and unfermented medium are indicated by an asterisk (*)

Likewise, the production of bacteriocins or exopolysaccharides during *Lb. paracasei* AF43 fermentation of WM cannot be excluded and may have contributed additional antimicrobial activity to fermented WM. Importantly, nutrient availability was not limiting in fermented WM. Residual lactose remained and, in unfermented medium, supported the onset of *L. monocytogenes* growth, confirming that carbon sources were available and that sugar depletion did not account for the observed inhibition.

Fermented WM contained the highest concentration of peptides in both the 6-3 kDa and <3 kDa ranges, fractions known for their antimicrobial potential. While peptides were also released in fermented PM and WPM, their antimicrobial effect may have been weaker, absent, or masked by the presence of readily metabolizable glucose. Furthermore, the more intense proteolysis observed in these two media may have degraded biologically active peptides into fragments with reduced or no antimicrobial activity. This interpretation is consistent with previous studies showing that, in some cases, extensive hydrolysis can reduce the biological activity of peptides (Solieri et al., 2022). Peptide-mediated antimicrobial activity arises from their ability to bind to the bacterial membrane via electrostatic interactions, thereby destabilizing the membrane and increasing its permeability. This deformation can lead to pore formation, allowing uncontrolled influx of molecules into the cell, ultimately impairing microbial metabolism, suppressing growth, and leading to cell lysis (Elsaadany et al., 2024). Importantly, this membrane-targeting mechanism also explains the differential susceptibility of bacterial groups: Gram-negative bacteria are less affected because their outer membrane provides an additional protective barrier that limits peptide access to the cytoplasmic membrane, whereas Gram-positive bacteria, lacking this defense, remain more vulnerable (Torcato et al., 2013).

5.4 Conclusions

This study provides a comprehensive characterization of the growth dynamics, metabolic adaptations, and functional properties of *Lb. paracasei* AF43 grown in whey protein, pea protein, and their combination. Integrating proteomics, targeted metabolomics, and biological activity assays revealed that the growth medium strongly shaped the strain's physiology and the bioactivity of the resulting fermentates. The strain exhibited a facultative homo-/heterofermentative profile, shifting from mainly homolactic fermentation in whey - driven by lactose hydrolysis and the tagatose-6-phosphate/Embden-Meyerhof-Parnas routes with prominent Ldh activity - to a phosphoketolase-driven heterofermentative metabolism in pea proteins, with mixed behavior in the hybrid media where preferential glucose uptake supported a predominantly homolactic output. This metabolic flexibility affected organic-acid patterns with potential functional impact: homolactic fermentation in whey and in the hybrid favored milder acidification. At the same time, acetate formation and citrate synthesis in pea may support the development of more complex flavor notes and redox

properties. This metabolic plasticity also influenced growth efficiency, and proteomic analysis played a key role in uncovering the underlying mechanisms. Ribosomal and central-carbon pathways were strongly activated in PM and WPM, supporting faster adaptation and growth, while WM required a metabolic investment in lactose/galactose utilization. These features favored milder proteolytic activity and the accumulation of low-molecular-weight peptides in fermented WM, particularly those below 3 kDa, known for their functional outcome. While fermentation performance is often evaluated in terms of rapid acidification and biomass accumulation, in this case, these features did not necessarily translate into improved health-promoting outcomes, highlighting that functional value may rely on complex metabolic adaptations. According to the substrate, *Lb. paracasei* AF43 drove specific biological properties: in WM, all three activities were expressed, consistently with higher peptide accumulation and possible peptide-mediated biological effects; in PM, the strongest effect was on FRAP, attributable primarily to citrate accumulation rather than peptide release; in WPM, fermentation markedly enhanced ACE-inhibitory activity, emphasizing the value of combining plant- and dairy-derived proteins. The ability of this strain to tailor antioxidant, ACE-inhibitory, and antimicrobial properties depending on the substrate underscores its potential as a candidate for the development of functional FFs.

5.5 Supplementary Material

Table S5.1 List of protein groups involved in carbohydrate metabolism expressed by *Lb. paracasei* AF43 cultivated in pea medium (PM), whey medium (WM), and the mixed medium (WPM) during the stationary phase of growth. Only protein groups showing statistically significant differences among conditions (p<0.05) are reported

Name	Intensity (log_2 LFQ)		
	PM	WM	WPM
LacA	$14.72^{b} \pm 0.87$	$21.94^{a} \pm 0.18$	$15.42^{b} \pm 2.70$
LacB	$19.27^{b} \pm 0.44$	$21.99^{a} \pm 0.06$	$16.39^{c} \pm 1.39$
LacC	$16.04^{b} \pm 0.76$	$19.54^{a} \pm 0.48$	$17.67^{b} \pm 1.05$
LacD	$18.85^{b} \pm 0.42$	$23.32^{a} \pm 0.33$	$18.84^{b} \pm 0.63$
Pts-Lac	$18.21^{b} \pm 0.64$	$19.37^{a} \pm 0.35$	$16.98^{ab} \pm 3.34$
Ldh	$24.20^{a} \pm 0.42$	$24.33^{a} \pm 0.35$	$23.27^{b} \pm 0.22$
Zwf	$18.37^{a} \pm 0.59$	$16.21^{b} \pm 0.71$	$18.51^{a} \pm 0.51$
Pgl	$17.45^{ab} \pm 1.59$	$18.17^{a} \pm 0.48$	$16.12^{b} \pm 0.2$
Gnd	$18.75^{a} \pm 0.75$	$16.71^{b} \pm 0.66$	$16.37^{b} \pm 0.79$
Xfp	$19.31^{a} \pm 0.47$	$17.85^{b} \pm 0.37$	$18.13^{b} \pm 0.63$
AckA	$18.43^{a} \pm 0.48$	$17.56^{b} \pm 0.15$	$16.54^{b} \pm 0.79$
PtsG	$21.58^{a} \pm 0.34$	$19.78^{c} \pm 0.54$	$20.88^{b} \pm 0.17$
Gap	$25.54^{a} \pm 0.11$	$24.33^{b} \pm 0.05$	$25.18^{a} \pm 0.30$
Pgk	$24.05^{a} \pm 0.16$	$22.55^{c} \pm 0.11$	$22.99^{b} \pm 0.11$
GpmA	$22.83^{a} \pm 0.07$	$21.44^{b} \pm 0.22$	$22.39^{ab} \pm 1.00$
Eno	$23.81^{a} \pm 0.25$	$23.31^{b} \pm 0.05$	$23.55^{ab} \pm 0.21$
Pyc	$17.48^{b} \pm 0.09$	$18.06^{a} \pm 0.16$	$17.58^{ab} \pm 0.65$
CS	$19.1^{a} \pm 1.24$	$16.51^{b} \pm 0.7$	$15.75^{b} \pm 1.04$

Statistical difference (p<0.05) within each media is indicated by different letters

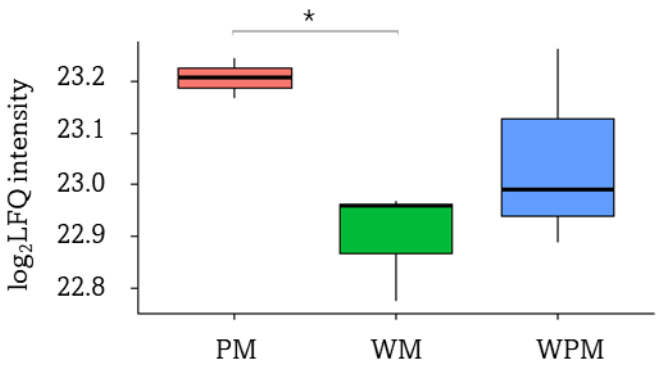

Figure S5.1 Glutathione metabolism expression (log_2 LFQ intensity) of *Lb. paracasei* AF43 cultivated in pea medium (PM), whey medium (WM), and the mixed medium (WPM). Asterisks (*) indicate statistically significant differences between conditions (p<0.05)

Chapter 6:
A Proteomic Approach for Unraveling the Effect of *In Vitro* Digestion on the Metabolism of *Lb. paracasei*

6.1 Introduction

Fermented foods (FFs) are increasingly recognized as functional foods due to their ability to promote human health. Their beneficial effects can be attributed to three main features: (i) the reduction of toxic and undesirable compounds, (ii) the generation of bioactive compounds during fermentation, such as peptides with functional properties, and (iii) the delivery of live microorganisms able to reach the colon. Among these microorganisms, lactic acid bacteria (LAB) represent the dominant group responsible for lactic fermentations. LAB can exert health-promoting effects by transiently colonizing the gut, competing with pathogenic bacteria, enhancing immune responses, and supporting a balanced intestinal microbiota (Mukherjee et al., 2024).

To exert such effects, however, ingested LAB must survive the harsh physicochemical and enzymatic conditions encountered throughout the gastrointestinal tract. During digestion, they are exposed to multiple stressors, including low pH and pepsin in the stomach, bile salts and pancreatic enzymes in the small intestine, as well as osmotic and oxidative stress, along with nutrient limitations. Moreover, antimicrobial peptides and host immune factors can further compromise bacterial survival (Castro-López et al., 2023). To withstand these conditions, LAB activate complex stress response mechanisms, including changes in transcription and protein expression, induction of stress-related metabolic pathways, reinforcement of cellular membranes, and production of protective metabolites. These adaptive processes are strongly influenced by the environmental conditions experienced before ingestion, particularly the growth conditions, which can shape the physiological state of the cells (Mills et al., 2011). Indeed, previous studies have demonstrated that growth conditions can significantly affect the tolerance of bacterial strains to gastrointestinal stress. For instance, Succi et al. (2017) showed that *Lacticaseibacillus rhamnosus* strains pre-cultivated in media containing different prebiotics as the sole carbon source exhibited variable survival rates when subjected to simulated gastrointestinal transit. These differences were attributed to physiological changes induced by the cultivation medium, which modulated the expression of stress-related proteins and enhanced resistance to acidic and bile conditions.

Proteomics has emerged as a powerful tool to study such adaptive responses at the cellular level (Stastna, 2024). Previous studies have investigated microbial adaptation to single stressors such as low pH or bile salts (Morales-Amparano et al., 2019). Although these approaches have provided valuable insights, they are limited by their simple design and do not fully capture the complexity of digestive transit. To the best of our knowledge, no studies have explored the effects of *in vitro* simulated digestion systems that mimic the sequential exposure of microorganisms to gastric and intestinal conditions, such as the standardized INFOGEST model (Brodkorb et al., 2019), and how these systems impact the microbial proteome. Addressing this gap may yield novel insights into the dynamic, integrated responses of bacteria under physiologically relevant conditions.

As described in Chapter 5, *Lacticaseibacillus paracasei* AF43 displayed distinct metabolic profiles and functional outcomes when cultivated in different protein-based media, indicating that the growth environment markedly influenced its physiological state. Building on these findings, the present study aimed to determine whether such pre-cultivation conditions also affect the strain's adaptive response during gastrointestinal transit. To this end, *Lb. paracasei* AF43 was grown in various protein media, used as models of fermented beverages from a sustainable perspective. Cells were then harvested and subjected to sequential gastric and intestinal phases following the standardized INFOGEST protocol. Proteomic changes were analyzed through label-free LC-MS/MS to identify key proteins and metabolic pathways involved in survival and adaptation. A comprehensive analysis of these proteomic changes will deepen our understanding of the adaptive strategies employed by *Lb. paracasei* AF43 under digestive stress, providing valuable insights for the design of functional FFs with enhanced bacterial resistance and health-promoting potential.

6.2 Materials and Methods

6.2.1 Materials

All materials used in this study are listed in Sections 2.2.1, 3.2.1, and 5.2.1

6.2.2 Media Preparation and Culture Conditions

Pea medium (PM), whey medium (WM), and a hybrid medium (WPM) were prepared as described in Section 5.2.2. The *Lb. paracasei* AF43 culture was reactivated and prepared as described in Section 5.2.2.

6.2.3 PM, WM, and WPM Fermentation by *Lb. paracasei* AF43

The *Lb. paracasei* AF43 culture was inoculated in PM, WM, and WPM (final concentration approximately 10^6 cfu/mL) and incubated at 30 °C until reaching the stationary phase (14 h for PM and WPM, and 48 h for WM, as determined in Chapter 5). Cell pellets from the stationary growth phase were collected by centrifugation (5,000 × g, 5 min, 4 °C). The resulting pellets were washed twice, resuspended in 1 mL of Ringer's solution, and subjected to *in vitro* digestion.

6.2.4 *In vitro* Digestion

In vitro digestion was performed on the cell pellets using the INFOGEST protocol (Brodkorb et al., 2019) as described in Section 3.2.5.1. At the end of the gastric phase, gastric digesta were cooled on ice. In contrast, at the end of the intestinal phase, intestinal digesta were added with Pefabloc® (final concentration: 5 mM) to inhibit enzymatic activity. Bacterial enumeration was carried out before digestion (UND) and at the end of both gastric (G) and intestinal (I) samples using MRS agar as detailed in Section 3.2.3.1.

All samples were centrifuged (Avanti™ J-25, Beckman Coulter, Brea, CA, USA) at 11,000 × g for 10 min at 4 °C to separate pellet and supernatant fractions. The resulting pellets were washed twice with Ringer's solution (5,000 × g, 5 min, 4 °C) and used for LC-MS/MS proteomic analysis.

6.2.5 Microbial Proteome Profiling Analysis by LC-MS/MS

Proteomic analysis was performed as described in Section 5.2.6, including protein digestion and purification (Section 5.2.6.1), label-free liquid chromatography-tandem mass spectrometry (LC-MS/MS) proteomics (Section 5.2.6.2), and data analysis (Section 5.2.6.3).

6.2.6 Statistical analysis

All trials were carried out in triplicate, and values were expressed as the means ± standard deviation. Statistical analysis was performed using R v 4.3.0 (The R Foundation for Statistical Computing, Vienna, Austria). Bartlett's test was used to check the homogeneity of variance. The difference between means was calculated using a t-test and one-way analysis of variance (ANOVA), and the Tukey test was used to determine statistically significant differences among means (p<0.05).

6.3 Results and Discussion

Lb. paracasei AF43 was cultivated in three protein-based media designed as models of sustainable fermented beverages with health-promoting potential: PM, WM, and their 50:50 combination, WPM. As described in Chapter 5, growth in these different environments markedly influenced the strain's metabolism and the health-promoting outcome of the resulting fermentates. Building on these findings, the present work aimed to assess whether such pre-cultivation conditions also affected the strain's performance under gastrointestinal stress. To this end, the survival of *L. paracasei* AF43 grown in the three protein-based media was first evaluated during simulated gastrointestinal digestion (Figure 6.1). Bacterial counts were determined before digestion (UND), after the gastric phase (G), and after the intestinal phase (I).

Cells grown in WM didn't show significant reductions throughout digestion, maintaining 8.72 ± 0.19 log cfu/mL after the intestinal phase, thus demonstrating a strong tolerance to simulated gastrointestinal conditions. Similar results were previously observed in Chapter 3, where this strain maintained high viability during digestion after being cultivated for 72 h in WM, further confirming its robustness in this environment. In contrast, cells grown in PM and in WPM were more affected, displaying a marked decrease (p<0.05) in viability during *in vitro* gastrointestinal digestion. In PM, counts decreased from 8.80 ± 0.17 log cfu/mL (UND) to 6.41 ± 1.25 log cfu/mL after the intestinal phase, corresponding to a reduction of more than 2 log units. Similarly, in the WPM, cells started from 8.99 ± 0.05 log cfu/mL, dropped to 7.72 ± 0.16 log cfu/mL after gastric digestion (approximately 1.5 log cfu/mL reduction), and remained stable afterward (7.58 ± 0.09 log cfu/mL in I),

indicating that the intestinal phase did not further impact viability. These results suggest that exposure to the acidic gastric environment represented the most critical challenge for *Lb. paracasei* AF43 survival when cultivated in PM and WPM. This is consistent with previous reports indicating that low gastric pH is one of the strongest selective pressures encountered by bacteria during gastrointestinal transit, often leading to significant loss of viability (Castro-López et al., 2023; Wu et al., 2011). Survival depends not only on the strain's intrinsic stress tolerance but also on the physiological state acquired during growth. Indeed, the composition of the growth medium can modulate stress resilience by influencing the accumulation of intracellular energy reserves, membrane composition, and stress-related proteins (Chen et al., 2025; Lv et al., 2017), which may have occurred in WM, thereby improving tolerance during digestion.

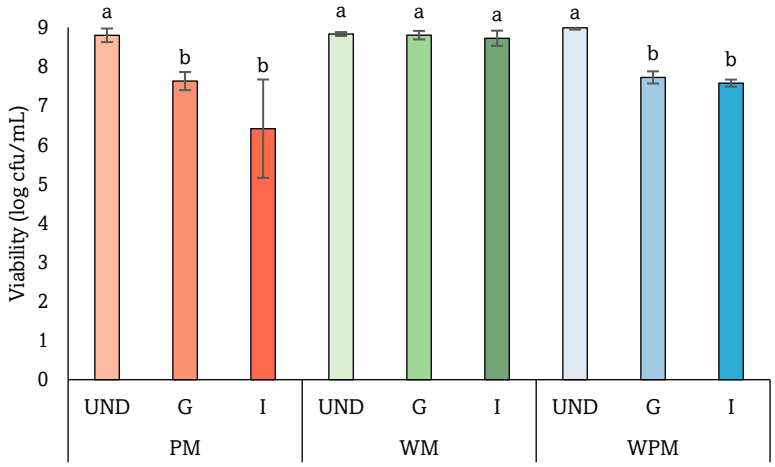

Figure 6.1 Viability (log cfu/mL; mean ± standard deviation) of *Lb. paracasei* AF43 before (UND), after gastric (G), and after intestinal (I) digestion phases. Different letters indicate statistically significant differences (p<0.05) among digestion phases within each media

The differential survival patterns observed provided the foundation for subsequent proteomic analyses, aimed at identifying how growth conditions influence the mechanisms underlying adaptation to gastrointestinal stress. Thus, label-free LC–MS/MS proteomic profiling was performed. A total of 1,489 protein groups were identified across all experimental conditions. Among them, 435 protein groups showed statistically significant differences in abundance between at least two conditions. The HPLC–MS/MS signal intensities were converted to LFQ values, which represent the relative abundance of each protein across samples (Van Den Bossche et al., 2025). Using KEGG KO annotation, the identified proteins were assigned to 102 distinct metabolic pathways.

First, ribosome metabolism was investigated, since it represents a key indicator of the physiological state of the microorganism: under favorable conditions, cells upregulate ribosome synthesis to support rapid growth; under nutrient limitation or stress, they downshift ribosomal investment and employ resources to protective functions (Cheng-Guang & Gualerzi, 2021; Scott et al., 2014). The expression of ribosome-related proteins in *Lb. paracasei* AF43 varied depending on both the digestion phase and the growth substrate (Figure 6.2).

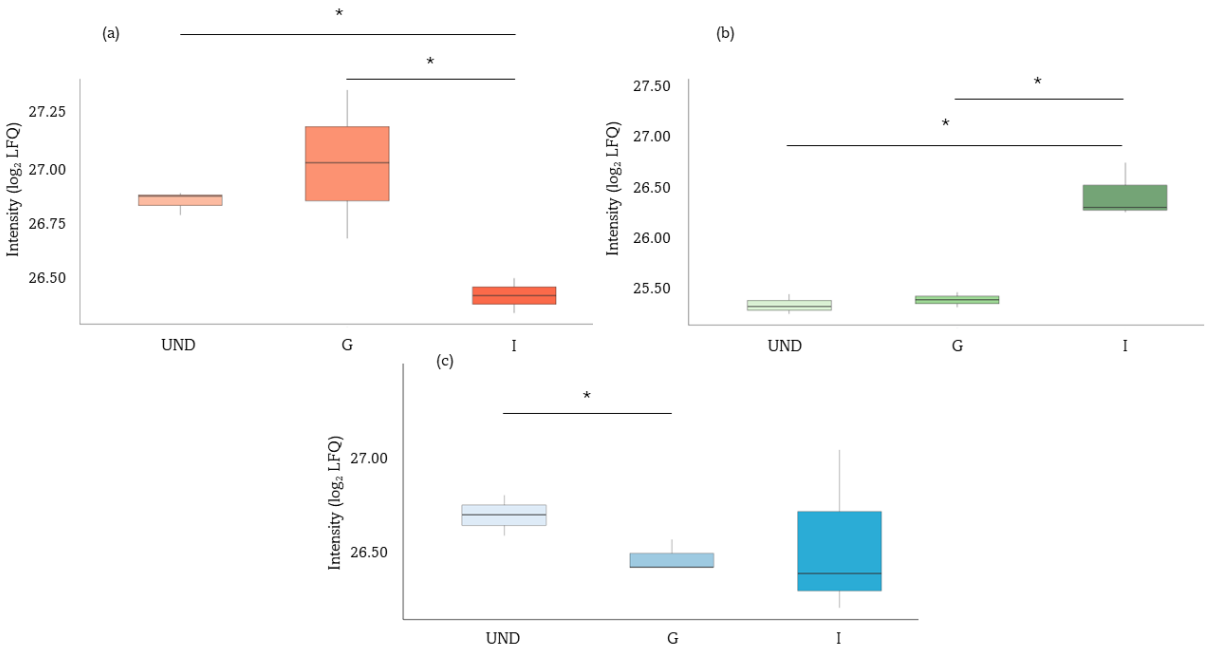

Figure 6.2 Intensity (log_2 LFQ) of ribosome metabolism detected in *Lb. paracasei* AF43 cultivated in (a) pea medium (PM, red), (b) whey medium (WM, green), and (c) mixed medium (WPM, blue) across different digestion phases: undigested (UND), after gastric phase (G), and after intestinal phase (I). Asterisks (*) indicate statistically significant differences between digestion phases within the same medium (p<0.05)

At the undigested stage, cells grown in PM and in WPM showed higher ribosome intensities (around 27 log_2LFQ), while those cultivated in WM started from lower levels (around 25.5 log_2LFQ) (p<0.05). This pattern is consistent with the faster growth observed in PM and WPM and the slower growth in WM, as described in Chapter 5. During the gastric phase, *Lb. paracasei* AF43 cultivated in PM showed a non-significant yet noticeable increase in ribosome metabolism, accompanied by high variability among replicates. This trend likely reflected a boost in translational capacity to synthesize stress-response proteins (e.g., chaperons) upon acid exposure, before energy-saving processes were activated (Broadbent et al., 2010). Following the intestinal phase, PM exhibited a significant decrease (p<0.05) compared to the gastric stage, indicating suppression of ribosomal function under sequential acidic and bile stress.

In contrast, WPM showed a significant reduction in ribosomal protein intensity after the gastric phase, suggesting rapid downregulation of this metabolic pathway as an energy-conserving response to acidity. Such a decrease in ribosome-associated proteins is a well-established adaptation strategy in LAB to harsh gastrointestinal conditions (Wu et al., 2011). Thereafter, ribosomal protein groups in WPM remained relatively stable through the intestinal phase, although with substantial variability among replicates. Such intra-population heterogeneity, also observed in PM, is well documented in bacterial stress physiology: single-cell studies have shown that bacterial populations often adopt heterogeneous strategies under stress, with subpopulations differing in metabolic state (Alnahhas & Dunlop, 2023). Notably, cells grown in WM, which initially exhibited low ribosome

intensity, showed no significant change between the undigested and gastric stages, consistent with a culture not primarily oriented toward rapid growth before digestion and thus requiring minimal adjustment in ribosome metabolism during this stage. Notably, a significant increase in ribosomal intensity was observed following the intestinal phase, suggesting that the cells restarted protein production and, possibly, growth once conditions became more favorable. This behavior may indicate that the WM helped *Lb. paracasei* AF43 to keep its protein-making machinery intact and to quickly reactivate translation when the acid stress decreased.

To better understand these changes in ribosomal protein group expression, carbon metabolism was examined. It involves the central pathways that convert sugars into ATP and reducing cofactors (NADH and NADPH), which are essential for growth, biosynthesis, and survival under changing environmental conditions (Papadimitriou et al., 2016). As shown in Figure 6.3, the overall pattern of carbon metabolism in *Lb. paracasei* AF43 was consistent across the three protein-based media.

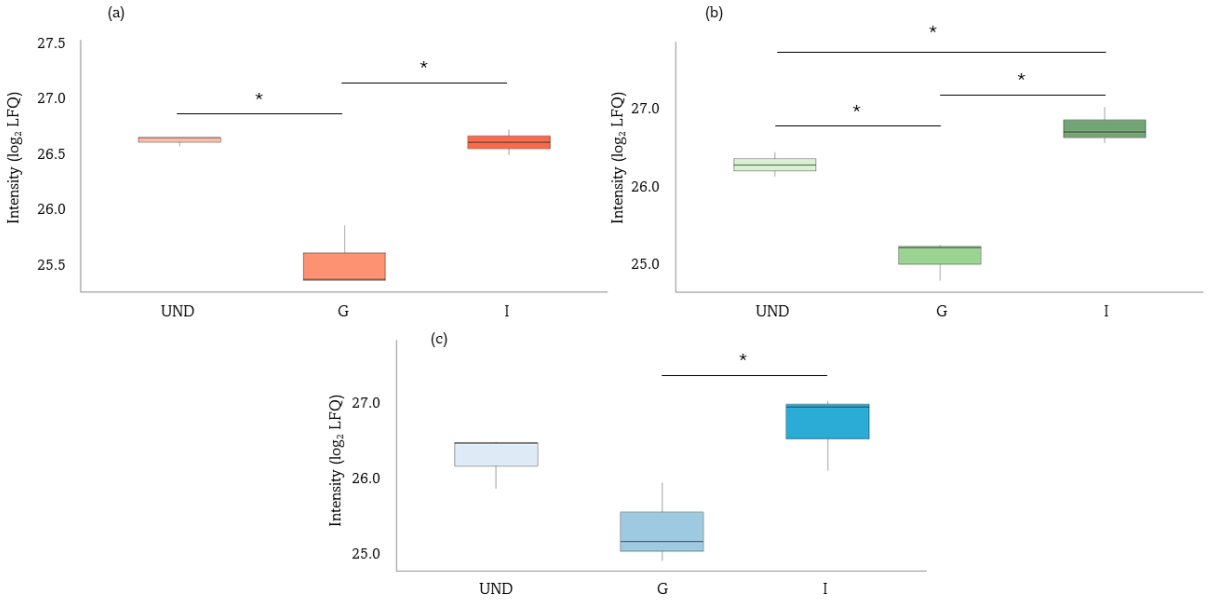

Figure 6.3 Intensity (log_2 LFQ) of carbon metabolism detected in *Lb. paracasei* AF43 cultivated in (a) pea medium (PM, red), (b) whey medium (WM, green), and (c) mixed medium (WPM, blue) across different digestion phases: undigested (UND), after the gastric phase (G), and after the intestinal phase (I). Asterisks (*) indicate statistically significant differences between digestion phases within the same medium (p<0.05)

All cultures exhibited a marked decrease (p<0.05) in the intensity of carbon metabolic protein groups during the gastric phase. Once again, the reduction in carbon metabolism under gastric stress suggests a shift from growth-oriented to energy-conservation metabolism, likely to allocate resources toward protective responses such as protein repair and membrane stabilization (Njenga et al., 2023).

During the intestinal phase, *Lb. paracasei* AF43 showed a recovery of carbon metabolism, indicating that bile stress was less severe than the acidic challenge faced during the gastric phase. Indeed,

bile salts generally exert a moderate stress on LAB, triggering adaptive metabolic modulation rather than a complete shutdown of cellular activity (Koskenniemi et al., 2011; Ruiz et al., 2013). This metabolic reactivation could help with different purposes depending on the growth substrate. In WM, where ribosomal proteins and other growth-related pathways (e.g., fatty acid biosynthesis; data not shown) also increased, the recovery likely reflected the reactivation of growth under intestinal conditions. In contrast, in PM, where ribosomal activity dropped below undigested stage levels (Figure 6.2), the enhanced carbon metabolism probably supported survival functions, providing energy for repair mechanisms rather than biomass production.

The suppression of carbon metabolism under stress has been previously reported in *Lactococcus lactis* and *Bifidobacterium longum*, where glycolytic enzyme activity was reduced to minimize ATP consumption, while F_1F_0-ATPase activity increased to maintain intracellular pH homeostasis (Carvalho et al., 2013a; Papadimitriou et al., 2016). Indeed, among the many mechanisms that LAB employ to counteract stress damage, the F_1F_0-ATPase system plays a central role in proton homeostasis. Table 6.1 shows the intensity of protein groups involved in the F_1F_0-ATPase system detected in *Lb. paracasei* AF43 in the different conditions tested.

This multimeric membrane enzyme hydrolyzes ATP produced during carbohydrate fermentation or free amino acid catabolism to pump protons out of the cytoplasm, generating a proton motive force and stabilizing intracellular pH. Under favorable conditions, the F_1F_0-ATPase can synthesize ATP by using the proton motive force. Still, under acid stress, it operates mainly by hydrolyzing ATP to expel protons and protect cytoplasmic enzymes from denaturation. The complex consists of two domains: the cytoplasmic F_1 sector (subunits AtpA, AtpB, AtpD, AtpG), responsible for ATP hydrolysis/synthesis, and the membrane-embedded F_0 sector (subunits AtpE, AtpF, AtpH, AtpC) that translocates protons across the membrane, linking ATP turnover to proton transport for pH homeostasis and metabolic efficiency (Papadimitriou et al., 2016).

In PM and WPM, only AtpD and AtpF were significantly upregulated during the gastric phase, and their levels returned to near baseline levels in the intestinal phase. AtpD, part of the system that drives ATP hydrolysis, and AtpF, a membrane-anchoring subunit that stabilizes the proton channel, are both essential for active proton removal. Their upregulation detected after the gastric phase likely reflected a response aimed at expelling excess protons and restoring intracellular pH during acid exposure (De Angelis & Gobbetti, 2004). The return to basal levels in the intestinal phase indicates that proton pumping was no longer prioritized once the pH stress was alleviated. This limited, reversible activation suggests that cells grown in PM and WPM adopt a conservative strategy, mobilizing only the most essential components of the ATPase complex to ensure survival.

Table 6.1 Intensity (log_2 LFQ; mean ± standard deviation) of F_1F_0-ATPase system subunits detected in *Lb. paracasei* AF43 cultivated in pea medium (PM), whey medium (WM), and mixed medium (WPM) at different digestion phases: undigested (UND), after gastric phase (G), and after intestinal phase (I)

	PM			WM			WPM		
	UND	G	I	UND	G	I	UND	G	I
AtpA	16.12 ± 2.22	13.31 ± 0.94	14.61 ± 2.91	17.2^{a} ± 0.28	13.91^{b} ± 0.67	14.89^{ab} ± 2.46	15.88 ± 1.96	13.75 ± 2.42	14.71 ± 1.66
AtpB	13.88 ± 2.03	13.52 ± 0.56	13.56 ± 1.52	15.85^{a} ± 0.45	12.26^{b} ± 1.03	13.68^{ab} ± 1.73	14.06 ± 0.74	12.57 ± 1.5	13.66 ± 0.36
AtpC	18.62 ± 0.39	17.3 ± 2.3	16.35 ± 1.93	17.69 ± 0.44	16.17 ± 2.63	14.87 ± 2.29	16.49 ± 0.95	16.9 ± 0.69	15.13 ± 3.11
AtpD	20.25^{b} ± 0.09	20.81^{ab} ± 0.54	21.86^{a} ± 0.06	21.22^{b} ± 0.07	21.76^{ab} ± 0.74	22.85^{a} ± 0.68	20.57^{b} ± 0.66	20.82^{b} ± 0.28	21.9^{a} ± 0.32
AtpE	13.02 ± 0.86	13.13 ± 0.65	12.8 ± 1.31	12.53 ± 0.38	13.17 ± 0.99	13.15 ± 1.03	15.07 ± 2.07	13.16 ± 0.75	13.62 ± 0.86
AtpF	18.45^{b} ± 0.18	21.98^{a} ± 0.33	16.3^{b} ± 1.93	16.5^{b} ± 0.64	20.53^{a} ± 1.5	16.92^{b} ± 2.37	15.06^{b} ± 0.53	20.44^{a} ± 1.15	16.02^{b} ± 1.16
AtpG	19.00 ± 0.92	19.95 ± 0.15	19.38 ± 0.27	17.59^{c} ± 0.30	19.48^{a} ± 0.24	18.6^{b} ± 0.27	18.69 ± 1.16	19.28 ± 0.82	17.41 ± 1.88
AtpH	19.15 ± 0.56	17.77 ± 4.05	18.53 ± 0.86	18.95 ± 0.22	19.42 ± 1.29	20.11 ± 1.37	18.99 ± 1.06	19.33 ± 0.79	17.07 ± 2.25

Statistical differences ($p<0.05$) among digestion phases within the same medium are indicated by different letters

In contrast, the response in WM was more extensive, involving both the catalytic and structural parts of the complex. During the gastric stage, AtpA and AtpB, which form the catalytic core, decreased significantly, possibly indicating a slowdown in ATP binding and hydrolysis to avoid unnecessary energy loss under harsh acid stress. At the same time, AtpF and AtpG increased markedly, suggesting activation of the proton channel (subunit F) and its coupling rotor (subunit G) to sustain efficient proton efflux. Following the intestinal stage, AtpD increased, accompanied by a slight increase in AtpA and AtpB, indicating that ATP hydrolysis resumed once the environment became more favorable. This may serve a dual purpose: maintaining proton balance in the presence of bile salts, which can disrupt membrane integrity and cause proton leakage (Bron et al., 2006), and regenerating ATP by reactivating the complex. Indeed, ATPase upregulation has been reported in lactic acid bacteria under bile stress, serving as a key mechanism for maintaining pH balance and recovering energy (Kaur et al., 2017). In WM, the F_1F_0-ATPase response appeared more adaptive and growth-oriented, as its recovery at the intestinal stage was accompanied by reactivation of carbon and ribosomal metabolism. This pattern indicates that *Lb. paracasei* AF43 not only endured digestion but also reactivated energy production and ribosomal activity once conditions became favorable. In contrast, the limited activation observed in PM and WPM suggests a survival-oriented strategy, where energy was directed toward essential proton regulation rather than growth.

Beyond energy balance and proton homeostasis, bacterial survival also relies on the activation of dedicated stress-response systems that safeguard cells from gastrointestinal transit (Mills et al., 2011). To investigate these additional adaptation strategies, protein groups associated with stress responses were analyzed. Several stress-related protein groups were identified in the proteome of *Lb. paracasei* AF43, and many of them showed differential expression among growth media and digestion phases (Table S6.1). To simplify interpretation, the results were cumulated, and the overall pattern is summarized in Figure 6.4a.

At the undigested stage, cells grown in WM displayed the highest cumulative intensity of stress-related protein groups, followed by those in PM and WPM. The higher baseline stress response metabolism in WM ($p<0.05$) may reflect the slower growth and lower ribosome activity observed in this medium (Chapter 5), suggesting that the strain adopted a more stress-resistant physiological state even before exposure to digestion. Conversely, in PM and WPM, which grew more rapidly, the strain likely prioritized growth-related functions, leading to lower expression of stress-related protein groups. During the gastric phase, all cultures showed an overall increase in cumulative stress response. However, the increase was modest in WM, while it was more pronounced in PM and WPM, consistent with the greater acid sensitivity observed in these two conditions (Figure 6.1). The stronger induction of stress-related protein groups in PM and WPM likely represents a response to severe acid stress, mobilizing repair and protection mechanisms to counteract protein

denaturation and membrane damage (Castro-López et al., 2023). In contrast, the strain in WM may have required a smaller upregulation to handle the acidic environment.

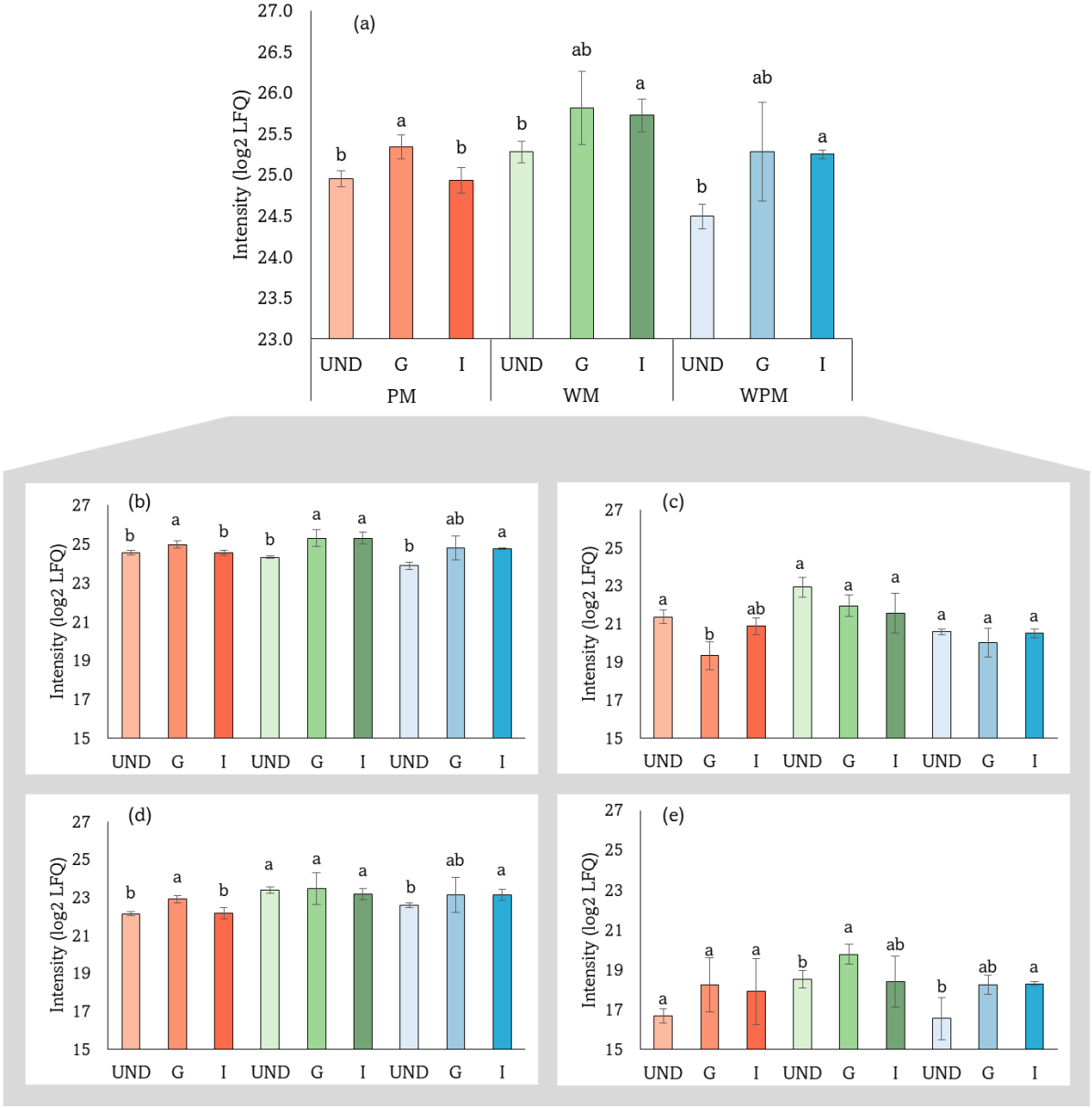

Figure 6.4 Cumulative expression levels (log_2 LFQ; mean ± standard deviation) of protein groups involved in stress-related pathways detected in *Lb. paracasei* AF43 cultivated in pea medium (PM; red), whey medium (WM; green), and mixed medium (WPM; blue) at different digestion stages (UND = undigested, G = after gastric phase, I = after intestinal phase). (a) Total stress response (sum of all stress-related protein groups); (b) chaperones and proteases; (c) universal stress proteins; (d) oxidative stress response; (e) osmotic stress response. Different letters indicate statistical differences (p<0.05) among digestive phases within the same medium

These observations are consistent with the concept that exposure to sub-lethal or stressful conditions can enhance bacterial robustness through physiological pre-adaptation. Recent studies have demonstrated that mild stress during cultivation can activate stress-response pathways, leading to increased resistance to subsequent challenges such as acid or bile exposure (Bisson et al., 2023; Marino et al., 2021). Such *active induction* of stress tolerance mechanisms, including chaperone activity, membrane remodeling, and redox regulation, has been proposed as an effective strategy

to enhance bacterial survival and function in dynamic environments such as the gastrointestinal tract (Chen et al., 2025).

Following the intestinal phase, the cumulative stress-response expression in PM returned close to the starting level, indicating that cells downregulated energy-demanding stress functions once the pH challenge was relieved, consistent with their reduced ribosomal and ATPase activity. By contrast, WM and WPM maintained elevated stress responses, particularly WM, where expression remained the highest among all conditions. This sustained activation suggests ongoing protective needs under bile exposure, which likely contributed to the higher survival rate observed in this condition.

To gain a deeper understanding of the mechanisms involved in stress-response, the identified stress-related proteins were classified into four functional categories: (i) chaperones and proteases, (ii) universal stress proteins, (iii) oxidative response protein groups, and (iv) osmotic response protein groups (Figure 6.4b-e). These categories represent the major physiological routes through which *Lb. paracasei* AF43 faced stress during gastrointestinal transit.

Chaperones and proteases are involved in the refolding or removal of misfolded and damaged proteins that accumulate under harsh conditions, such as exposure to acid and bile (Castro-López et al., 2023; Gobbetti et al., 2015). In all media, this category significantly increased (p<0.05) during the gastric phase, confirming a strong induction of the acid shock response. In WM and WPM, high levels were maintained throughout the intestinal phase, whereas in PM, they declined once the stress was relieved. The main proteins contributing to this trend were ClpX, DnaJ, DnaK, GrpE, FtsH, GroL, and HtrA (Table S6.1), all of which are known molecular chaperones or ATP-dependent proteases (Mills et al., 2011). Similar activation of these systems under acid and bile stress has been widely reported in LAB, where GroEL and DnaK promote refolding of denatured proteins, while Clp and FtsH complexes degrade irreversibly damaged ones, maintaining protein homeostasis (Gobbetti et al., 2015; Papadimitriou et al., 2016). HtrA and FtsH also play protective roles at the membrane interface, ensuring stability during bile exposure (Savijoki et al., 2005). Moreover, the persistence of high expression in WM during the intestinal phase may reflect the need for continued protein turnover and membrane maintenance. At the same time, cells resume growth, in line with the elevated ribosomal and metabolic activity observed in this medium.

Universal stress proteins, which act as general protectants that enhance tolerance to multiple stresses during growth (Kvint et al., 2003), showed only minor fluctuations across conditions. However, in PM, they decreased significantly during the gastric phase, suggesting that cells under severe acid stress prioritized targeted protective mechanisms (e.g., chaperones and ATPase) rather than a universal stress response.

The oxidative stress-related class, including protein groups such as CydC, Nox, TrxA, TrxB, LplA, and GshAB, showed distinct patterns across media. In WM, levels remained stable throughout

digestion, whereas in PM they increased significantly during the gastric phase and returned to baseline afterward. In contrast, WPM showed a slight increase during the gastric phase, which persisted into the intestinal phase (p<0.05). Although typically linked to oxidative stress, these proteins are also responsive to acid-induced oxidative imbalance. This mechanism has been reported in several *Lactobacillus* species exposed to acid or bile, where redox stress arises from proton influx and membrane perturbation (Gobbetti et al., 2010; Mills et al., 2011). For instance, thioredoxins (TrxA, TrxB) and glutathione-related enzymes (GshAB) detoxify reactive oxygen species and protect cysteine residues in metabolic enzymes under low pH (Papadimitriou et al., 2016). Likewise, LplA enhances redox defense, binding lipoic acid, which acts as a cofactor for antioxidant enzymes (Cronan, 2024).

Finally, proteins associated with the response to osmotic stress showed an increasing trend during digestion in all media. This pattern is consistent with the osmotic challenges imposed by both acidic conditions and bile salts, which disturb ionic balance and membrane permeability, potentially compromising cell integrity throughout gastrointestinal transit (Papadimitriou et al., 2016).

6.4 Conclusions

This work demonstrates that the nutritional environment preceding gastrointestinal exposure plays a decisive role in shaping the stress tolerance of *Lb. paracasei* AF43. Cells cultivated in WM, despite slower growth rates, developed a more stress-resistant phenotype, resulting in higher survival under simulated gastrointestinal conditions. This enhanced robustness was associated with a metabolic state characterized by reduced ribosomal investment during growth, sustained basal activation of stress-related pathways, and a flexible F_1F_0-ATPase response capable of maintaining intracellular homeostasis under acid and bile stress. In contrast, cells grown in PM or WPM, which promoted faster growth and higher ribosomal activity before digestion, were more susceptible to acidic conditions, suggesting that rapid growth was achieved at the expense of stress resilience. Overall, these findings strengthen the concept that pre-cultivation conditions act as a form of physiological pre-adaptation.

Proteomic data provided mechanistic insight into these differential adaptive behaviors. Under gastric conditions, all cultures showed a transient suppression of growth-associated metabolisms (e.g., ribosomal and carbon pathways), coupled with the activation of protective mechanisms aimed at preserving energy and maintaining protein and membrane integrity. In particular, the upregulation of F_1F_0-ATPase subunits highlighted the central role of proton efflux systems in mitigating cytoplasmic acidification. Concurrently, the induction of chaperones and proteases reflected a coordinated strategy to repair or remove denatured proteins under harsh stress conditions.

Future studies should aim to link these findings to specific physiological outcomes, such as membrane fluidity, bioactive metabolite production (e.g., antioxidants), and host interaction, as well as to test whether such effects persist *in vivo*.

Ultimately, understanding how the cultivation environment shapes bacterial resilience provides a powerful tool for designing FFs that deliver live, metabolically active microorganisms capable of withstanding digestive transit and exerting positive effects at the gut level.

Table S6.1 Intensity (log_2 LFQ) of stress-related proteins detected in *Lb. paracasei* AF43 cultivated in pea medium (PM), whey medium (WM), and mixed medium (WPM) and collected before digestion (UND), after gastric phase (G), and after intestinal phase (I). Proteins are grouped into functional categories: chaperones and proteases, universal stress proteins, oxidative stress response, and osmotic stress response

	PM			WM			WPM		
	UND	G	I	UND	G	I	UND	G	I
Chaperons and proteases									
ClpB	$17.08^{b} \pm 0.22$	$15.28^{c} \pm 0.37$	$18.28^{a} \pm 0.14$	17.24 ± 0.1	16.18 ± 1.78	17.14 ± 2.61	15.48 ± 1.85	15.99 ± 1.97	16.48 ± 2.51
ClpC	21.41 ± 0.53	21.52 ± 0.71	21.63 ± 0.32	$21.54^{b} \pm 0.15$	$22.53^{a} \pm 0.37$	$22.55^{a} \pm 0.46$	$20.39^{b} \pm 0.19$	$21.58^{a} \pm 0.6$	$21.8^{a} \pm 0.71$
ClpE	18.74 ± 0.83	20.17 ± 0.71	18.5 ± 0.13	$18.94^{b} \pm 0.26$	$21.71^{a} \pm 0.16$	$20.68^{a} \pm 0.63$	18.59 ± 1.11	20.06 ± 1.09	18.9 ± 0.54
ClpP	21.5 ± 0.46	22.38 ± 0.99	20.93 ± 0.19	$20.02^{b} \pm 0.5$	$21.49^{ab} \pm 1.32$	$22.22^{a} \pm 0.62$	$18.33^{b} \pm 0.35$	$21.67^{a} \pm 1.22$	$20.71^{a} \pm 1.17$
ClpX	$17.31^{b} \pm 0.43$	$19.25^{a} \pm 0.47$	$18.63^{ab} \pm 1.06$	$17.34^{b} \pm 0.62$	$19.38^{a} \pm 0.93$	$19.02^{a} \pm 0.65$	$17.97^{a} \pm 0.03$	$18.67^{ab} \pm 0.8$	$20.46^{a} \pm 1.47$
DnaJ	$17.51^{b} \pm 0.55$	$20.47^{a} \pm 0.85$	$15.6^{c} \pm 0.07$	$17.2^{b} \pm 0.12$	$19.77^{a} \pm 0.69$	$17.19^{b} \pm 0.81$	$17.04^{ab} \pm 1.92$	$20.21^{a} \pm 1.93$	$15.81^{b} \pm 1.05$
DnaK	22.06 ± 0.42	22 ± 0.69	22.81 ± 0.45	$21.35^{c} \pm 0.21$	$22.03^{b} \pm 0.25$	$22.92^{a} \pm 0.08$	$21.54^{b} \pm 0.23$	$22.00^{a} \pm 0.16$	$22.54^{ab} \pm 0.78$
GrpE	$17.9^{b} \pm 0.84$	$20.24^{a} \pm 0.79$	$19.49^{ab} \pm 0.31$	$19.4^{b} \pm 0.25$	$21.05^{a} \pm 0.82$	$20.1^{ab} \pm 1.03$	18.05 ± 2.2	20.67 ± 1.00	18.74 ± 0.9
FtsH	$20.83^{a} \pm 0.25$	$20.62^{a} \pm 0.18$	$19.58^{b} \pm 0.49$	$20.05^{b} \pm 0.18$	$21.24^{a} \pm 0.09$	$20.17^{b} \pm 0.49$	19.6 ± 0.39	20.03 ± 0.46	19.26 ± 1.18
GroL	$22.89^{a} \pm 0.25$	$21.79^{b} \pm 0.16$	$22.89^{a} \pm 0.12$	$22.51^{b} \pm 0.09$	$22.31^{b} \pm 0.23$	$23.6^{a} \pm 0.19$	$22.37^{b} \pm 0.07$	$21.60^{c} \pm 0.04$	$23.12^{a} \pm 0.43$
GroS	18.91 ± 0.13	17.45 ± 1.98	19.56 ± 0.55	20.53 ± 0.14	19.43 ± 4.49	20.64 ± 0.92	17.53 ± 0.36	19.08 ± 2.37	17.54 ± 3.1
HrcA	14.69 ± 1.16	15.67 ± 1.18	13.95 ± 0.03	15.47 ± 0.69	15.76 ± 0.39	14.88 ± 1.25	13.85 ± 1.25	16.18 ± 1.19	14.46 ± 1.4
HtrA	$21.51^{b} \pm 0.04$	$23.04^{a} \pm 0.46$	$19.62^{c} \pm 0.33$	21.84 ± 0.13	22.94 ± 1.07	21.03 ± 0.95	21.27 ± 0.95	22.35 ± 1.98	20.73 ± 0.64
Universal stress proteins									
Usp1	$16.11^{a} \pm 0.33$	$14.06^{b} \pm 0.11$	$17.03^{a} \pm 0.36$	$17.92^{a} \pm 0.73$	$13.26^{b} \pm 0.84$	$15.62^{ab} \pm 2.07$	$15.72^{a} \pm 0.36$	$14.38^{ab} \pm 0.9$	$13.96^{b} \pm 0.48$
Usp5	$17.48^{a} \pm 0.63$	$14.93^{b} \pm 0.62$	$15.77^{ab} \pm 1.86$	$18.84^{a} \pm 0.26$	$16.51^{b} \pm 0.85$	$14.76^{b} \pm 2.34$	16.59 ± 1.01	16.52 ± 1.56	16.13 ± 1.08
UspA	17.68 ± 1.22	18.89 ± 0.93	17.29 ± 0.49	$18.45^{b} \pm 0.22$	$20.7^{a} \pm 0.83$	$19.18^{ab} \pm 0.61$	16.56 ± 0.68	17.86 ± 1.18	16.42 ± 1.31
Usp1	$21.07^{a} \pm 0.42$	$16.68^{b} \pm 0.68$	$20.59^{a} \pm 0.39$	$22.72^{a} \pm 0.56$	$21.08^{b} \pm 0.44$	$20.99^{ab} \pm 1.58$	20.31 ± 0.24	19.3 ± 0.83	20.28 ± 0.34

	PM			WM			WPM		
	UND	G	I	UND	G	I	UND	G	I
Oxidative response									
CydC	13.16^{b} ± 0.76	16.34^{a} ± 0.93	11.81^{b} ± 1.1	12.9 ± 0.63	12.07 ± 1.21	13.64 ± 0.75	13.41 ± 1.4	13.88 ± 1.66	13.74 ± 1.62
CydD	12.77 ± 1.67	13.49 ± 0.51	12.59 ± 1.83	13.27 ± 1.03	12.89 ± 0.65	13.18 ± 0.88	13.29 ± 0.86	13.95 ± 0.78	13.89 ± 0.82
Nox	12.64 ± 1.07	13.24 ± 0.65	13.44 ± 1.25	12.07^{b} ± 0.29	14.38^{a} ± 1.03	14.14^{ab} ± 3.02	12.37 ± 1.72	13.54 ± 0.7	15.68 ± 2.89
Npr	21.47^{a} ± 0.24	18.8^{b} ± 0.92	21.49^{a} ± 0.41	23.12^{a} ± 0.21	20.67^{b} ± 0.55	22.71^{a} ± 0.5	22.22^{a} ± 0.16	20.66^{b} ± 0.92	22.78^{a} ± 0.33
Pox	14.6 ± 0.97	14.24 ± 0.66	14.17 ± 0.44	12.51 ± 0.23	15.76 ± 2.1	14.88 ± 1.87	14.27 ± 0.9	15.91 ± 1.8	14.39 ± 0.61
TrxA	15.79^{c} ± 0.73	22.35^{a} ± 0.35	18.19^{b} ± 0.01	18.26^{b} ± 0.45	22.34^{a} ± 1.43	19.61^{b} ± 0.81	16.34^{b} ± 0.91	21.85^{a} ± 2.1	19.26^{a} ± 0.38
TrxB	16.72^{b} ± 0.9	18.94^{a} ± 0.21	18.09^{a} ± 1.35	18.9 ± 0.2	20.02 ± 1.28	18.92 ± 0.63	17.85^{b} ± 0.48	19.36^{a} ± 0.44	17.89^{b} ± 0.24
LplA	16.69^{b} ± 0.59	18.94^{a} ± 1.25	16.76^{ab} ± 0.54	16.82^{b} ± 0.23	20.65^{a} ± 0.64	15.81^{b} ± 1.68	17.19 ± 0.94	18.46 ± 0.89	14.99 ± 2.27
GshR	20.36^{a} ± 0.15	19.1^{ab} ± 0.78	19.73^{b} ± 0.08	19.75^{a} ± 0.41	18.92^{b} ± 0.19	19.21^{ab} ± 0.53	19.56 ± 0.64	18.57 ± 0.67	19.7 ± 0.33
GshR3	14.03 ± 0.89	14.99 ± 0.94	16.01 ± 1.85	16.45 ± 0.44	16.04 ± 3.31	17.19 ± 2.09	13.67 ± 1.24	14.34 ± 1.41	14.14 ± 0.35
GshAB	12.47^{b} ± 0.73	13.91^{a} ± 0.28	13.24^{ab} ± 0.69	12.97 ± 1.09	13.7 ± 1.24	13.31 ± 0.35	12.6 ± 1.81	13.85 ± 1.92	14.29 ± 1.13
Osmotic response									
OpuCA	15.41 ± 0.49	15.02 ± 0.73	16.59 ± 1.01	17.31 ± 0.35	17.76 ± 1.19	17.31 ± 2.33	14.89 ± 1.69	15.45 ± 1.49	17.27 ± 0.66
OpuCD	14.03 ± 0.68	15.94 ± 2	12.74 ± 0.18	13.78^{b} ± 2.29	17.61^{a} ± 0.66	13.08^{b} ± 0.91	13.31 ± 0.3	14.61 ± 2.73	13.51 ± 0.91
OpuCC	14.52 ± 0.69	15.87 ± 2.24	16.32 ± 2.57	17.23 ± 0.92	18.29 ± 0.97	16.4 ± 1.03	13.95^{b} ± 1.76	17.28^{a} ± 0.3	15.84^{ab} ± 1.51
OpuCB	12.94 ± 0.81	14.22 ± 0.53	14.7 ± 2.61	12.85 ± 2.13	14.64 ± 2.41	14.16 ± 0.33	12.92 ± 1.79	13.18 ± 0.43	14.77 ± 1.38
GbuA	13.26 ± 0.52	16.36 ± 1.89	12.87 ± 0.75	12.13 ± 0.27	13.06 ± 1.61	13.6 ± 1.09	13.54 ± 1.16	13.43 ± 1.14	13.79 ± 1.4

Statistical differences ($p<0.05$) among digestion phases within the same medium are indicated by different letters

General Conclusions and Future Perspectives

The overall aim of this PhD project was to explore the potential of autochthonous lactic acid bacteria (LAB) to generate health-promoting properties from food proteins through fermentation. By combining microbiological, biochemical, and omics-based analyses within a single experimental framework, this research provided an integrated understanding of how lactic fermentation can be exploited as a biotechnological strategy to develop functional and sustainable fermented foods (FFs) from both animal and plant protein sources.

The selection process identified LAB strains capable of producing protein hydrolysates with relevant biological activities, including antioxidant, ACE-inhibitory, and antimicrobial properties. Nevertheless, the outcomes demonstrated that these effects were highly dependent on both the microbial strain and the protein substrate, highlighting the importance of a strain-matrix specificity in driving the functional properties of the resulting fermented products. Overall, whey protein fermentation enabled robust, broadly distributed bioactivities across several LAB strains, confirming whey as a favorable substrate for functional fermentation. Within this context, *Lb. paracasei* AF43 clearly emerged as the most promising strain, consistently contributing to all three bioactivities investigated, highlighting its strong potential for the development of whey-based fermented products that combine nutritional value with bioprotective properties. In contrast, in the pea protein system, bioactive properties were more strongly strain-dependent and less uniformly expressed. The fermentate obtained with *Lb. brevis* C9 exhibited the highest radical-scavenging activity, whereas *Lb. plantarum* M2 showed the highest reducing power and, together with *Leuc. pseudomesenteroides* I3, exerted consistent antimicrobial effects against Gram-positive bacteria. This strain- and substrate-dependent variability suggests that, depending on the desired health-promoting function, targeted combinations of microbial strains and protein substrates can be strategically designed to tailor the nutritional and functional attributes of FFs.

Another key outcome of this work is that protein-based fermentates can significantly influence gut microbial ecology. Although proteins are rarely investigated for their impact on the gut microbiota compared to carbohydrates and are often associated with detrimental fecal fermentations, this study revealed that selected LAB-derived fermentates positively modulated intestinal microbial communities. Specifically, several fermentates promoted health-associated taxa such as *Bifidobacterium longum* and *Akkermansia muciniphila*, while enhancing metabolic pathways associated with gut homeostasis and SCFA production. These results highlight that, under appropriate fermentation conditions, protein-derived substrates can serve as multifunctional dietary components that simultaneously provide nutritional, functional, and microbiota-modulating benefits. In this regard, several whey-derived fermentates, especially those produced by *Lb. delbrueckii* strains, were effective in driving these positive ecological shifts, reinforcing the relevance of this group for the development of whey-based fermented products with targeted microbiota-modulating potential. Among pea-based fermentates, *Leuc. citreum* C10 emerged as the fermentate with the most

consistent positive impact on gut microbial composition, selectively promoting probiotic and commensal taxa such as *Bifidobacterium longum* and *Parabacteroides distasonis*, while reducing pro-inflammatory species including *Bilophila wadsworthia*. In contrast, fermentate derived from *Lb. plantarum* M2 was particularly effective at sustaining beneficial microbial metabolites, such as butyrate and formate, while also supporting the growth of less desirable taxa, underscoring the complex, matrix-dependent nature of microbiota responses to pea-protein fermentation.

Finally, the in-depth characterization of *Lacticaseibacillus paracasei* AF43 revealed how the nutritional environment profoundly shapes microbial metabolism, functional outputs, and stress responses. In whey-based media, a slower and more controlled fermentation favored an improved bioactivity of the fermentate and enhanced physiological robustness, resulting in improved tolerance to gastrointestinal stress. Conversely, fermentation in plant-based or hybrid media induced distinct metabolic and proteomic profiles, reflecting substrate-specific adaptive mechanisms that can be exploited to fine-tune the bioactivity of fermented products.

From a methodological standpoint, integrating classical microbiology with proteomic and metagenomic approaches proved instrumental for dissecting microbial adaptation and functionality. This framework not only advanced current understanding of LAB-protein interactions but also established a methodological basis for the rational design of protein-based FFs that combine nutritional quality, microbial resilience, and health-promoting potential.

The findings of this PhD project open several promising directions for future research. The protein-based systems developed in this work were conceived as model platforms for the formulation of fermented beverages and functional foods intended for human consumption. Building on these results, future research should aim to bridge laboratory-scale experimentation with product development, ensuring functionality, stability, and consumer acceptability.

A primary objective will be to identify and sequence the peptides generated during fermentation. These analyses will help clarify whether the observed bioactivities can be attributed to specific BPs or to the synergistic effect of peptide mixtures. Further attention should also be devoted to the identification of other molecules within the fermented matrices that may contribute to the bioactivities, such as exopolysaccharides, bacteriocins, and phenolic compounds. A comprehensive evaluation should also include assessing the stability and bioaccessibility of these metabolites during *in vitro* gastrointestinal transit to determine whether the compounds produced can retain their structural integrity and biological activity under physiological conditions. Subsequent *in vivo* studies will be essential to confirm the health-promoting potential of the most promising fermentates.

From a technological perspective, the next step should involve the formulation and optimization of a prototype fermented beverage derived from the most efficient strain-substrate combinations

identified in this study. The resulting product should be evaluated not only for its biochemical and functional attributes but also for its sensory profile and consumer acceptability. If necessary, formulation adjustments or process refinements can be implemented to improve sensory quality without compromising the functional performance of the product.

Although this thesis has only partially explored hybrid protein systems, combining animal and plant proteins, these represent a particularly promising frontier for future development. Such blends could exploit the complementary amino acid profiles and techno-functional properties of both sources, potentially improving texture, stability, and nutritional balance. Moreover, fermentation of these hybrid matrices may generate novel peptide signatures and metabolite patterns, expanding the functional and sensory diversity of next-generation FFs.

Another key aspect for the practical application of LAB-based protein fermentates concerns the assessment of functional stability during storage, which remains a critical requirement for industrial translation. In particular, it will be necessary to monitor how microbial viability, peptide integrity, and metabolite composition evolve over time under different storage and processing conditions, as these factors directly affect functional efficacy, product safety, and quality consistency. Potential degradation of bioactive peptides, shifts in metabolic profiles, or loss of viable LAB during storage may compromise the reproducibility of the functional properties observed at the laboratory scale. Moreover, these changes can lead to alterations in the product's sensory characteristics, such as taste, aroma, and texture, potentially affecting consumer acceptance.

In parallel, scaling LAB-based protein fermentation from laboratory to industrial level represents a decisive step to evaluate technological robustness, process control, and economic feasibility. Key challenges include maintaining strain-specific functional performance across batches, controlling fermentation parameters in complex protein matrices, and integrating downstream processing steps such as product standardization and shelf-life preservation into existing industrial workflows. Addressing these limitations through pilot-scale trials and process optimization will be essential to define sustainable large-scale production strategies, ultimately supporting the development of innovative fermented beverages that effectively combine nutritional functionality, consumer acceptability, and environmental sustainability.

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Rossi, A., Porcellato, D., Innocente, N., & Marino, M. A proteomic approach for unraveling the effect of *in vitro* digestion on the metabolism of *Lb. paracasei* AF43. Manuscript in preparation.

Other Relevant Techno-Scientific Publications

Innocente, N., Marino, M., Renoldi, N. & **Rossi, A.** (2024). "Rafforzamento della tipicità e miglioramento della sostenibilità della filiera produttiva del formaggio Montasio DOP". Consorzio per la tutela del formaggio Montasio, Università degli Studi di Udine, Regione Autonoma Friuli Venezia Giulia. Gr Grafiche, Rovigo, Italy.

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*personally delivered

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