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Lactic Acid Bacteria as Bioprotective and Health-Promoting Cultures in the Food Industry

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“Courage doesn’t always roar.
Sometimes courage is the little voice at the end of the day that says
I’ll try again tomorrow.”

Mary Anne Radmacher

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Abstract

In this PhD thesis, the potential of lactic acid bacteria (LAB) as bioprotective and health-promoting cultures in the food industry was investigated.

The first part of this PhD thesis was focused on the bioprotective activity of LAB in seafood products. The foodborne pathogen *Listeria monocytogenes* intentionally inoculated in cold-smoked sea bass was effectively inhibited by one of the LAB cultures tested. The product inoculated with LAB did not lead to the development of any off odor and off flavour. Some of the LAB cultures tested resulted to be also able to control the spoilage microflora of sea bass and sea bream burgers, avoiding bloating spoilage. This results in an extension of the shelf-life of fish burgers, which, as it is well known, are highly perishable. In this case, bioprotective cultures had produced some changes in the sensory profile of the product, however, the inoculated burgers resulted to be preferred by the sensory panel compared to the control (without LAB inoculation). These results demonstrated the potential use of bioprotective cultures to improve the quality and safety of fishery products, contributing to the reduction of food waste in the fish supply chain.

The second part of this PhD thesis was focused on the study of the probiotic properties of several strains belonging to *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei* and *Lacticaseibacillus rhamnosus* species, previously isolated and characterized. Many of the tested strains showed potential probiotic properties, in particular, they were able to efficiently adhere to intestinal epithelial cells which is an essential requirement to play a beneficial role in the intestinal tract. Heat-killed (HK) cells and cell-free supernatants (CFS) derived from several probiotic strains were tested for their potential antiproliferative activity against colorectal cancer cells. Results revealed that HK cells did not have any significant effect on cancer cell viability. On the contrary, a significant antiproliferative effect of the CFS extracellular metabolites produced by all the strains was observed, regardless of pH and in a time-dependent manner. This constitutes a starting point for the future individuation and characterization of the metabolite/s is/are responsible for the antiproliferative effect. Understanding the mechanism involved in the reduction of cell viability is also fundamental for the potential application of these compounds for therapeutic applications. The ability of

LAB to produce membrane vesicles (MVs). Probiotic-derived MVs are gaining interest for their possible use in the treatment of diseases using them as therapeutic carriers since evolving evidence is demonstrating their beneficial effects on human health. Two type strains belonging to *L. paracasei* and *L. rhamnosus* species resulted to be able to produce a considerable amount of MVs which were subsequently characterized. The size distribution of MVs was measured using two different techniques (Nanoparticle Tracking Analysis and Atomic Force Microscopy). LpMVs resulted to have a lower size compared to those produced by *L. rhamnosus*, although the formers were higher in number. Fatty acids analysis revealed that, according to the literature, MVs derive from the cytoplasmic membrane of Gram-positive bacteria since the main abundant FAs were the same for both MVs and parental bacterial cells. Proteins and RNA quantifications demonstrated that these two classes of components are present in MVs, and, in the future, proteomic and transcriptomic analyses will be performed with the aim to contribute to understanding the biological function of MVs. The association of beneficial effects to the cellular components or extracellular metabolites of probiotic bacteria will limit, at least in part, the use of the live probiotics, avoiding the risks associated with the administration of live organisms.

Part 1

Lactic Acid Bacteria
for
Food Quality and Safety

1. Introduction

1.1 Progress and trends that are shaping the future of food system

Ensuring food quality and safety remains the major challenge for the agri-food sector. Despite advances in food science, foodborne diseases are still a global burden, remaining unexpectedly high in both developed and developing countries (Todd, 2020). At the same time, ensuring the reduction of environmental and climate impact of the food system, making it more sustainable, is becoming a cornerstone in food production. One effective measure to reduce food waste along the food chain, from farm to fork, extending the shelf-life of perishable food products, while still guaranteeing food safety and high nutritional standards. Many different food additives have been developed over time to meet the needs of food production, they are needed to safeguard the safety of processed food and the maintenance of the good conditions throughout its journey from factories to consumers. However, this has its limitations. The association between certain additives and health-related issues and the consequent awareness for foods without synthetic additives has led to explore new ways to ensure the safety in extending the shelf-life (Sindelar & Milkowski, 2012). In the recent years, many efforts have been done to replace certain preservatives with natural and label-friendly alternatives, such as herbs and spices extracts, that are safer for consumers and the environment, since “green” consumerism is becoming more and more popular (Piskernik et al., 2011; Lara et al., 2011; Lorenzo et al., 2014; Azizkhani et al., 2016; Huang et al., 2021; Sterniša et al., 2020). Simultaneously, other new strategies have been developed and one of them originates from food fermentation, used since ancient civilization for preserving food. This practice has a huge influence on the organoleptic properties of food products since during fermentation microorganisms produce organic acids, ethanol, carbon dioxide, and several aromatic compounds. Moreover, it can improve the nutritional quality of food and the consumption of fermented food can be used for the delivery of beneficial microorganisms and bioactive compounds (Caplice & Fitzgerald, 1999). However, this influence is not always desired, especially when fresh-like foods with their natural nutritive values and sensory attributes are required by consumers. Over time, it was noticed that some microorganisms can be present in food without interfering with food taste and texture. The observation that this

type of microorganisms can also produce bacteriocins and other antimicrobial compounds paved the way toward bioprotection using microorganisms. Bioprotective culture is defined as live microorganisms that are deliberately added to food to control its bacteriological status without changing its technological, nutritional, and sensory quality (Martin et al., 2021). This approach holds much promise, also considering the ongoing trend of consumers to ask for clean label and minimally processed food.

1.2 Seafood: so precious, so perishable

Seafood, including various species of fish, mollusks, and crustaceans play an important role in the human diet as they are a source of valuable nutrients such as unsaturated fatty acids, proteins, vitamins, and minerals.

In particular, they contain high levels of polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which provide protection from cardiovascular diseases, cancer, and autoimmune disorders (Huynh & Kitts, 2009; Sidhu, 2003; Kapoor et al., 2021).

However, they can be a double-edged sword since they also constitute one of the major potential sources of foodborne illness as they can harbor different foodborne pathogens including *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli*, and *Campylobacter* spp. (Baptista et al., 2020).

Listeria monocytogenes is considered a serious concern in different kinds of refrigerated ready-to-eat (RTE) products, especially for its capability to grow at refrigeration temperatures (EFSA BIOHAZ Panel, 2018). Ready-to-eat fish products with extended storage time are associated with a high risk of transmission of this pathogen (Kramarenko et al., 2016). The presence of *L. monocytogenes* in these types of products have been documented by several authors, it was isolated from fried fish, barbecue cuttlefish, sushi, smoked salmon and trout, and seafood salad (Jamali et al., 2013; Garrido et al., 2009; Nakamura et al., 2004; Gombas et al., 2003). Listeriosis is still one of the most concerning food-borne diseases throughout the world due to the severity of manifestations (including septicemia, meningitis, and fetal death), with a case-fatality rate between 20% and 50% (Garrido et al., 2010).

Moreover, the high water activity (Aw), neutral pH, and relatively high content of low molecular weight nitrogenous compounds make seafood a highly perishable commodity susceptible to the development of spoilage bacteria (Etemadi et al., 2013). Spoilage bacteria constitute a significant issue for the food industry and lead to food loss and waste, which are translated also into economic losses. Although microbial activity is the main factor limiting the shelf-life of these types of products, it is worth noting that the initial loss of freshness is primarily related to the activity of endogenous enzymes, such as proteases, and lipid oxidation to which seafood lipids are particularly subjected, since they consist mainly of polyunsaturated fatty acids (Ghaly et al., 2010; Sriket, 2007).

Among the microbial community of seafood exists a small fraction, referred to as "specific spoilage organisms" (SSOs), to which is attributable the spoilage activity that is reflected in changes in the sensory properties of the product, through the production of amines, sulphides, alcohols, aldehydes, ketones, and organic acids, that makes it unsuitable for consumption (Gram et al., 2002). The identification of this group is fundamental for the development of preservation techniques. You need to know your enemy before you have any hope of defeating him. However, this constitutes a long-standing question due to the diversity and dynamics of SSOs that are selected by several factors including fish originating, processing, storage conditions, and microbial interaction (Boziaris & Parlapani, 2017; Saenz-García et al., 2020). Consequently, it is easy to understand that developing effective preservation strategies against seafood spoilage microorganisms is complex.

Ensuring the nutritional quality and safety and extending the shelf-life of these highly perishable foods have been the aim of different studies carried out in recent years and it will be for a long time to come. Foods are an extremely complex and dynamic system where different endogenous and exogenous factors interact with each other and are involved in the shaping of microflora composition. The spoilage microbiota of several seafood has been found to be dominated by pH-sensitive psychrotrophic Gram-negative bacteria *Shewanella* and *Pseudomonas*, which are particularly competitive at chilled storage temperatures (Françoise et al., 2010). The use of vacuum or modified atmosphere (MAP) inhibits the aerobe bacteria, resulting in a dominance of other bacteria including LAB, *Photobacterium phosphoreum*, *Brochothrix thermosphacta*, and *Vibrionaceae* under limited oxygen conditions (Gram and Dalgaard, 2002). However, *Shewanella putrefaciens*, along with other

marine bacteria, is able to use trimethylamine-N-oxide (TMAO) for anaerobic respiration as a terminal electron acceptor that allows the development even under oxygen-limited conditions, leading to the production of trimethylamine responsible for the rotten fish off-odours (Debevere & Boskou, 1996).

Given the complexity and unpredictability of seafood microflora, the simultaneous use of more than one preservation technique, namely hurdle technology, in these types of products turns out to be very effective.

1.3 Seafood bioprotection: Where are we now?

As mentioned before, food products are systems extremely complex and dynamic and these characteristics are reflected in their microbial ecology, as part of them. This inevitably leads each single microorganism to have some kind of interaction with the others that could in certain cases produce an antagonistic effect, for instance through the production of a wide array of metabolites that may negatively affect the growth of the others.

The bioprotection approach was developed based on this concept. It offers the potential to extend the shelf-life and improve food safety using microorganisms and therefore is becoming part of preservation strategies applied to seafood and seafood products, and beyond.

The microbial safety and stability of most food, including seafood and seafood products, are ensured by the application of the hurdle technology.

Although many different Gram-positive and Gram-negative species can have inhibitory activity by producing antimicrobial substances, including bacteriocins, lactic acid bacteria (LAB) are of particular interest to the food industry.

In this context, LAB can be considered a new generation of food additives. This can be linked to the fact that LAB are commonly part of several food products and their use in food industry as starter cultures and probiotics are widespread (Coelho et al., 2022). Lactic acid bacteria naturally dominates the microbiota of many foods from which they are frequently isolated and for this reason, they are presumably compatible with the product characteristics (Schroeter & Klaenhammer, 2009; Reina et al., 2005). Non-autochthonous isolates are not necessarily well adapted to the specific characteristics of the food matrix.

Moreover, some of them have the GRAS (Generally Recognized as Safe) or QPS (Quality Presumption Safety) status and therefore a full safety assessment is not required during the selection procedure of bioprotective culture (Ly et al., 2022).

Different *in vitro* and challenge test experiments have demonstrated that LAB, or their metabolites, are able to inhibit seafood spoilage and pathogen microorganisms. However, the effect of LAB in seafood can be controversial and must be assessed through *in situ* experiments.

To be used as bioprotective cultures, and hence be commercialized and used in the food industry, a strain must fulfill several requirements, for instance, it is required to grow well at refrigeration temperature as they have to compete with undesired psychrotrophic bacteria.

The antagonistic activities of bioprotective cultures can be exerted in different modes of action that are divided in passive (competition for space, nutrients, and oxygen) or active (production of antimicrobial substances) (Buchanan & Bagi, 1997; Chen et al., 2021; Castro et al., 2011; Ren et al., 2022; Bian et al., 2011).

However, in the food system, the antagonistic activity of LAB are mainly due to the competition for nutrients and the production of one or more antimicrobial compounds (Reis et al., 2012). The major and evident effect of LAB in food is the rapid acidification due to the production of organic acids, mainly lactic acid. Although the production of several acids by carbohydrate metabolism is a common feature of LAB, in contrast, the production of antimicrobial peptides like bacteriocins is not so widespread among this group (Castellano et al., 2008).

A particular reference to bacteriocin-producing strains is made when referring to bioprotection. Currently, bacteriocins are considered the young up-and-comer of food additives and constitute a hot area of research for their application as novel therapeutic agents in healthcare (Quintana et al., 2014; Naghmouchi et al., 2010).

Not all the bacteriocins discovered and studied revealed to be suitable for application in food, they often have a narrow activity spectrum and those produced by Gram-positive bacteria are generally active against closely related species and therefore inactive against Gram-negative bacteria, which constitutes the majority of the undesired microorganisms in seafood (Holtsmark et al., 2008).

For this reason, the impact of bacteriocinic activities on the total viable flora had to be taken into account. Their addition could create microbial imbalance and could favor the development of spoilage microorganisms.

They can be added to food in the form of catabolism products of a bacteriocin-producing strain or additives and each one brings with it pros and cons. The use of the latter form generally does not have an impact on the sensory properties of food and the ability of the bacteriocin-producing strain to grow and produce the bacteriocins in food is not required. However, it is more expensive and bacteriocins can be absorbed and degraded in the food matrix losing their activity (Aasen et al., 2003; Silva et al., 2018).

An additional strategy could be the incorporation of the bioprotective culture or bacteriocin into food packaging or coatings, improving the stability of the peptide and avoiding the potential negative effect on sensory properties of the direct inoculation of the starter culture (Angiolillo et al., 2018; Contessa et al., 2021).

Looking at the potential of the use of LAB in the fish industry, in Table 1.1 are summarized the studies where the use of bioprotective cultures in fish products was effective in reducing or inhibiting different undesired microorganisms.

As can be seen in Table 1.1, the bacteria most frequently used as bioprotective cultures are lactobacilli and *Carnobacterium*, which are also the genus of LAB most frequently isolated from seafood.

Carnobacterium is a genus of lactic acid bacteria known for comprising several bacteriocinogenic strains with bioprotective potentialities against different food-borne pathogens. Both *C. divergens* and *C. maltaromaticum* are able to grow in a wide variety of refrigerated raw and processed seafood products stored aerobically, vacuum-packaged or packaged under MAP conditions (Laursen et al., 2005).

The effective bioprotective activity is certainly species and strain dependent, however, in food, it may be subjected to a great variation compared to the majority of chemical preservatives. This can be due to the diversity of storage conditions of the food product and its physico-chemical and microbial composition which, for even the same type of products, can be constituted by different species and strains (Gursky et al., 2006; Himelbloom et al., 2001; Siedler et al., 2020). The technological processes, especially in small artisanal

production plants, have high variability and result in products of the same type (e.g., cold-smoked salmon) having different characteristics (Siddi et al., 2022).

This constitutes one of the main disadvantages of the use of microorganisms in food production, the complexity of the interaction with the food matrix and the related microbiota made the development of effective bioprotective cultures still a current challenge.

Table 1.1 Bioprotective cultures against pathogens and spoilage microorganisms in seafood and seafood products.

Bioprotective culture	**Isolation source**	**Inoculated product**	**Effect**	**References**
Lactobacillus sakei CTC494	meat-borne	vacuum-packed hot-smoked sea bream	Inhibition of *L. monocytogenes*	Bolìvar et al., 2021
Carnobacterium maltaromaticum CTC1741	cold-smoked salmon	vacuum-packed cold-smoked Atlantic salmon	Reduction of the growth of *L. monocytogenes* (< 2 Log increase)	Aymerich et al., 2019
Lactococcus lactis KF147 and *Lactobacillus plantarum* ATCC 14917 in co-culture *Lactococcus lactis* KF147 and *Carnobacterium piscicola* AT 71101238000999 in co-culture *Lactobacillus plantarum* ATCC 14917 and *Carnobacterium piscicola* AT 71101238000999 in co-culture *Lactococcus lactis* KF147, *Lactobacillus plantarum* ATCC 14917 and *Carnobacterium piscicola* AT 71101238000999 in co-culture	fresh fish	vacuum-packed fresh farmed sea bass fillets	Each LAB combination produced the inhibition of the growth of *L. monocytogenes* ATCC 070101121 and of the spoilage microflora of the product	Boulares et al., 2017
Lactococcus lactis KF147 and *Carnobacterium piscicola* AT 71101238000999 in co-culture	fresh fish	vacuum-packed fresh farmed sea bass fillets	Bacteriostatic effect against mesophilic aerobic plate counts (MAPC) and psychrotrophic bacterial counts (PBC)	Boulares et al., 2013
Lactobacillus paracasei IMPC 4.1	CNR-ISPA collection	tuna burgers packed under MAP	Increase of the shelf-life of the product by delay off-odour development	Danza et al., 2018

Lactobacillus sakei Lb706	Federal Center for Meat Research Kulmbach, Germany	vacuum-packed rainbow trout fillets	Decreasing of the growth rate of *L. monocytogenes*	Aras Hisar et al., 2005
Lactobacillus pentosus 39	naturally fermented Italian sausages	fresh salmon fillets packed in air	Reduction of the growth of *L. monocytogenes* ATCC 19117 and *Aeromonas hydrophila* ATCC 14715	Anacarso et al., 2014
Lactobacillus curvatus CWBI-B28	raw meat	cold-smoked salmon in air	Reduction of *L. monocytogenes* 22 counts	Ghalfi et al., 2006
Lactococcus lactis subsp. *lactis* KT2W2L	water of mangrove forest in Southern Thailand	cooked, peeled, and ionized tropiccal shrimps under MAP	Inhibition of *Brochothrix thermospacta* CD274 and *Listeria innocua* CIP	Hwanhlem et al., 2015
Carnobacterium divergens V41[1] and *Lactococcus piscium* CNCM I-4031[2] in co-culture	trout[1]; fresh MAP salmon steak[2]	cooked and peeled tropical shrimp packed under MAP	Lower *L. monocytogenes* count compared to the control; improved sensory quality in the early stage of storage	Saraoui et al., 2017
Lactococcus lactic ssp. *lactis*	Not Specified	vacuum packed raw Atlantic salmon	Shelf-life prolonged of three days	Ibrahim and Vesterlund, 2014
Pediococcus acidilactici	Fargo-35, Laboratorios Amerex S.A. (Madrid, Spain)	cold-smoked salmon in air	Reductionf of *L. monocytogenes* counts	Montiel et al., 2013
Lactobacillus plantarum IFRPD P15 and *Lactobacillus reuteri* IFRPD P17 in co-culture	naturally fermented plaa-som	Plaa-som fermented fish	Inhibition of *E. coli*	Saithong et al., 2010
Carnobacterium maltaromaticum SF1944	cold-smoked salmon	vacuum-packed salmon dill gravlax slices	Inhibition of spoilage microbiota and *L. monocytogenes*	Wiernasz et al., 2020

Lactobacillus plantarum 3; *Lactobacillus pentosus* 7	seafood products	vacuum-packed minced sea bass	Suppression of TVB-N and TMA production, presumabily due to the inhibition of spoilage microflora	El Bassi et al., 2009
Leuconostoc mesenteroides	lightly preserved minced herring product	minced yellowfin tuna	Reduction of the total bacterial counts and TVB-N, histamine, malonaldehyde values	Gelman et al., 2000

1.4 Seafood bioprotection: Where should we go?

Currently, the use of conventional preserving techniques for seafood is still needed and the use of bioprotective cultures should be considered only as an additional hurdle to good manufacturing, processing, storage, and distribution. In order to allow the implementation of this technology in the food industry, the effect of the selected LAB on seafood quality had to be further considered. Many studies have focused on the antimicrobial effect of microorganisms *in vitro*. Unfortunately, this is only one of the first steps in the development of a bioprotective culture and, although the results obtained from these preliminary studies may show inhibition against target organisms, the application *in situ* must be tested to confirm their effectiveness, as the bioprotective activity may differ greatly depending on the food matrix.

For instance, further investigative studies are required to define what are the mechanisms and the critical factors that regulate bacteriocin production in order to optimize their production and before simple pilot-scale studies can be scaled up into useful commercial biopreservative applications.

This lack of information hamper large-scale implementation at industrial level that can be easily demonstrated by the following data.

The majority of commercially available bioprotective cultures for food application are addressed to milk and dairy products and represent about the 60% of the total whereas those for seafood and seafood products constitute only the 3% (Fisher & Titgemeyer, 2023).

In this context, the selection of a bioprotective culture with the role of starter culture or probiotic, which deserves the name of “super microorganism”, will represent a great opportunity to substantially improve the food quality reducing the production costs. To date, this can be achieved more realistically by the simultaneous use of more than one culture.

Studies aiming to further investigate the behavior in food of the *in vitro* selected bioprotective cultures will be fundamental for this area of research.

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2. Analysis of the Bioprotective Potential of Different Lactic Acid Bacteria Against *Listeria monocytogenes* in Cold-Smoked Sea Bass, a New Product Packaged Under Vacuum and Stored at 6 ± 2 °C

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2.1 Introduction

Meat from seafood is an important source of high quality protein for humans (Tidwell & Allan, 2001). However, it is highly susceptible to both microbiological and chemical deterioration due to its high water activity (Aw), neutral pH, relatively high contents of nitrogen compounds and free amino acids and presence of autolytic enzymes (Etemadi et al., 2013). Rapid cooling and storage on ice extend the shelf-life of fish and fishery products to only 9–12 days, despite the use of innovative packaging and technological methods (Çoban et al., 2016). To extend the conservation of fresh fish, technological processes are increasingly used to decrease or inhibit degradation activity. Smoking is one of these techniques. Fish products such as salmon and trout are salted and cold-smoked to increase their shelf-life and organoleptic characteristics. Recently, to provide new products with high nutritional value, cold smoking of unconventional fish products has been used. In Friuli, a company expert in the smoking of fish products has investigated the potential of cold-smoking sea bass fillets. In Italy, sea bass are usually marketed in boxes with ice flakes or at 4 °C packaged in air or under vacuum, gutted and whole or as fillets. Consequently, their shelf-life is limited to 9–12

days. In fact, within a short time, tissue enzymes and contaminating microbial populations adapted to refrigeration temperatures, produce metabolites typical of spoilage, which consist of basic volatile nitrogen (ammonia compounds), trimethylamine, amines, peroxides, and compounds derived from the degradation of lipids (malonaldehyde; Joffraud et al., 2001; Comi, 2016; Iacumin et al., 2017, 2020). Therefore, smoking of trout, salmon, and other fish is increasingly used to extend their shelf lives up to 45–60 days under refrigeration. Sea bass, which is medium-sized fish comparable to trout and salmon, is cold-smoked at a maximum temperature of 29 °C. The technology applied is similar to that used for trout and salmon and includes: i) the selection of raw materials; ii) threading (baffe/fillets); iii) dry salting; vi) traditional cold smoking, without using additives; finishing of the threads; v) cutting, vacuum packing and storage at 4 °C; vi) delivery and sale of the finished product at 4 °C. Cold-smoked sea bass fillets can be included in the category of minimally processed foods, which are foods treated with techniques that preserve them but also retain to a greater extent their nutritional quality, sensory, and hygienic characteristics by reducing the reliance on heat as the main preservative action (Raybaudi-Massilia et al., 2013). Consumers usually prefer foods which retain their natural nutritional and sensory properties (Sillani & Nassivera, 2015). As mentioned before, cold-smoked fish are produced by minimal processing techniques that include salting, smoking, vacuum packaging and refrigeration to meet this challenge of replacing traditional methods of preservation whilst retaining nutritional, sensory and hygienic quality. The microbial ecology of cold-smoked fishery products has been intensively studied and consists of *Enterobacteriaceae*, *Shewanella putrefaciens*, *Aeromonas* spp., *Pseudomonas* spp., *Photobacterium phosphoreum*, lactic acid bacteria (LAB), and *Brochothrix* spp. (Hansen et al., 1996; Leroi et al., 1998, 2001; Hansen and Huss, 1998; Joffraud et al., 2001, 2006; Cardinal et al., 2004; Lyhs et al., 2007; Bernardi et al., 2009; Leroi, 2010). Lactic acid bacteria and *Brochothrix thermosphacta* grow preferable in both vacuum and modified atmosphere packaging (MAP) smoked fish (Laursen et al., 2005; Leroi, 2010). Unfortunately, also the pathogen *Listeria monocytogenes* can contaminate cold-smoked fish during the storage. Recent studies have evaluated the ability of LAB to control *L. monocytogenes* in food products such as cheese, sliced cooked cured pork shoulder, diced ham, fresh-cut lettuce, processed seafood, mayonnaise-based seafood salads, cold-smoked salmon, hot-smoked sea bream, and many others (Bredholt et al., 1999; Mataragas et al.,

2003; Allende et al., 2007; Mejlholm & Dalgaard, 2015; Aymerich et al., 2019; Bolìvar et al., 2021; Iacumin et al., 2020; Morandi et al., 2020). In particular, a 1.5–2.5 Log reduction of *L. monocytogenes* by the presence of bacteriocin-producing strains belonging to *P. acidilactici*, *L. sakei*, *L. plantarum*, and *L. curvatus* species was obtained in sausages. These bacteria could be used alone or in combination (Vignolo et al., 2015). Lactic acid bacteria exert their bioprotective activity against *L. monocytogenes* through production of organic acids or bacteriocins (Gàlvez et al., 2007; Zhou et al., 2015). These bacteriocin inhibition effects are likely caused by different actions, such as competition for nutrients, as well as organic acid and bacteriocin production, in addition to the "hurdle" parameters. Strains of *Lactobacillus sakei*, *L. casei*, *L. brevis*, *L. curvatus*, *L. plantarum*, and *Carnobacterium* spp. isolated from meat products frequently produce bacteriocins or bacteriocin-like compounds, and in particular, these strains have good antilisterial effects and therefore are used as bioprotective cultures in European meat products (Schillinger et al., 1991; Hugas et al., 1996; Kotzekidou & Bloukas, 1996; Bredholt et al., 2001; Leroy et al., 2006; Vignolo et al., 2015). The main advantage is that the inoculation of some LAB strains does not influence the quality of the final product with any sensory alteration. Considering that *L. monocytogenes* exhibits different levels of virulence and pathogenicity, several discriminatory typing methods have been described (Brosch et al., 1996; Wernars et al., 1996). Typing by pulsed-field gel electrophoresis (PFGE), which has thus far provided discrimination of strains, has rapidly become the standard typing method for detecting outbreaks of listeriosis (Brosch et al., 1996; Graves & Swaminathan, 2001). However, this method is laborious and time-consuming (Miettinen et al., 1999; Buchrieser et al., 1993; Farber & Harwing, 1996), lasting 4–5 days. Considering the above information, the aim of this work was to use bioprotective cultures to eliminate or reduce the growth of *L. monocytogenes* intentionally inoculated in cold-smoked sea bass.

2.2 Materials and methods

2.2.1 Substrate preparation and group subdivision

Three lots of sea bass were raised in sea cages by Orada adriatic d.o.o. in Cres, Croatia. They were collected, eviscerated, placed in polystyrene boxes containing ice, and sent to a

processing plant in the Friuli region within 5 h. This company has extensive experience in the cold smoking of fish products such as both farmed and wild salmon and, especially, trout. The fish were filleted (baffe) and salted to a water phase salt (WPS) value higher or equal to 3.5%, and then they were desalted and smoked at low temperature (<30 °C). After smoking, the fillets were vacuum-packed in plastic bags (PE/PA Niederwieser, Collecchio, Italy), stored at 4 °C and transported to the Department of Agricultural, Food, Environmental and Animal Sciences of the University of Udine. Each sample weighed approximately 200 g.

The samples of each lot were divided into 10 groups of 15 samples each, as follows, and analyzed in triplicate at 0, 15, 30, 45, and 60 days (that represents the typical deadline of the shelf-life of cold-smoked fish):

1. Control samples (not inoculated);
2. Samples inoculated with a mix of *L. monocytogenes* (LM);
3. Samples inoculated with Sacco LAK-23 (*Latilactobacillus sakei*) starter and a mix of *L. monocytogenes* (LM);
4. Samples inoculated with *Carnobacterium* (CB) spp. and mix of *L. monocytogenes* (LM);
5. Samples inoculated with *Lacticaseibacillus casei* (SAL 106) and a mix of *L. monocytogenes* (LM);
6. Samples inoculated with *Lacticaseibacillus paracasei* (SAL 211) and a mix of *L. monocytogenes* (LM);
7. Samples with inoculated LAK-23 (*Latilactobacillus sakei*);
8. Samples with inoculated *Carnobacterium* (CB) spp.;
9. Samples with inoculated *Lacticaseibacillus casei* (SAL 106);
10. Samples with inoculated *Lacticaseibacillus paracasei* (SAL 211).

2.2.2 Preparation of L. monocytogenes suspension

The inoculum consisted of three strains of *L. monocytogenes* derived from International Culture Collections and the Collection of the Department of Agricultural, Food, Environmental and Animal Sciences of the University of Udine. In particular, the following strains were used: *L. monocytogenes* NCTC 10887 (serotype 1/2b) and *L. monocytogenes* 9Di4a (serotype 4b) isolated from fish matrices and *L. monocytogenes* 11Di4a of human origin and responsible for invasive listeriosis. Single suspensions were prepared using a 3-

day *L. monocytogenes* cultures grown at 6 ± 2 °C on plate count agar (Oxoid, Italy) added to saline-peptone water (NaCl 3.5%, bacteriological peptone [Oxoid, Milan, Italy] 0.1%, distilled water; Aw 0.96) and the absorbance at an optical density of wavelength of 600 nm (A_{600}) was adjusted to 0.1. To evaluate the concentration of each suspension, equivalent dilutions were prepared using sterile saline-peptone water, and 0.1 mL of each dilution was surface cultured onto plates containing Palcam agar base (Oxoid, Milan, Italy). The plates were incubated at 37 °C for 48 h, and the grown colonies were counted. Each suspension contained approximately 7–8 Log CFU/mL.

2.2.3 Preparation of the L. monocytogenes suspension for the test samples

A cocktail (stock suspension) was prepared from suspensions containing the three different *L. monocytogenes* strains in saline-peptone water (NaCl 3.5%, bacteriological peptone 0.1%, distilled water; Aw 0.96; 7 Log CFU/ml). The stock suspension was diluted and inoculated by spreading 0.1 mL onto cold-smoked sea bass fillets (final value – approximately 2 Log CFU/g product).

2.2.4 Starter culture

One commercial and three selected starter cultures were used. All starters were bacteriocin producers, as "tested *in vitro*." The commercial starter culture was produced and sold by Sacco S.r.l. (Cadorago, Italy) and contained LAK-23 (*Latilactobacillus sakei*, bacteriocin producer) isolated from meat product. The culture was freeze-dried, packaged in a foil pouch, and stored frozen. At the time of use, it was thawed, homogenized and diluted in sterile saline-peptone water. To assess the culture concentration, dilutions were performed using sterile saline-peptone water, and 0.1 mL of each dilution was inoculated into Man, Rogosa, and Sharpe medium (MRS, Oxoid, Milan, Italy) by the double-layer method. The plates were incubated at 37 °C for 48–72 h, and the grown colonies were counted. The suspension contained approximately 11 Log CFU/g. The selected LAB starters, *Lacticaseibacillus casei* (SAL 106) and *Lacticaseibacillus paracasei* (SAL 211), were isolated from milk products (Iacumin et al., 2015). *Carnobacterium maltaromaticum* was recently isolated during a monitoring to determine the hygienic quality of "Montasio" cheese produced in Friuli. The *Lacticaseibacillus* strains were cultivated in MRS broth and *Carnobacterium* was cultivated

in TSM agar (Tryptic Soy Medium with 5% glucose, 2% NaCl and pH 8, Oxoid, Milan, Italy). Subsequently, the strains were harvested by centrifugation (9,700 x *g*) and then diluted in saline-peptone water to evaluate their concentrations. Decimal dilutions of all starter cultures were made, and then 0.1 mL was inoculated by spreading onto cold-smoked sea bass fillets (final value: approximately 5 Log CFU/g product).

2.2.5 Inoculated samples

For each test and lot, 15 smoked sea bass samples were inoculated and analyzed in triplicate at each time point: 0, 15, 30, 45, and 60 days. Fifteen samples were stored as originally packaged and represented the controls, and the others were unpackaged and inoculated with *L. monocytogenes* alone, with all starters alone and with the starters and *L. monocytogenes* mix and then repackaged according to the technique and packaging used by the facility. The control (uninoculated) and inoculated samples were stored at 6 ± 2 °C, that is the standard temperature of a supermarket refrigerator in Italy (AAVV, 2021).

2.2.6 Microbiological analyses

At the established dates, three samples from each group were subjected to microbiological analyses, which included evaluation of the total bacterial count (TBC) in Gelysate agar (gelatine sugar-free agar, Oxoid, Milan, Italy) incubated at 30 °C for 48–72 h; LAB in MRS agar incubated at 37 °C for 48 h (double layer method); *Carnobacterium* in TSM agar incubated at 30 °C for 2 days under anaerobiosis; yeasts and moulds in Malt Extract agar (MEA, Oxoid, Milan, Italy) incubated at 25 °C for 72–96 h; total coliforms and faecal coliforms in Violet Red Bile Lactose agar (VRBLA, Oxoid, Milan, Italy) incubated, respectively, at 37 and 44 °C for 24 h; coagulase-positive staphylococci in Baird–Parker agar medium (BP, Oxoid, Milan, Italy) with egg yolk tellurite emulsion added (Oxoid, Milan, Italy) incubated at 35 °C for 24–48 h and confirmed by the coagulase test; sulfite-reducing clostridia in Differential Reinforced Clostridial medium (DRCM, VWR, Avantor, Pennsylvania, USA) incubated at 37 °C for 24–48 h in a jar prepared for anaerobic reaction with a gas-packing anaerobic system (BBL, Becton Dickinson, United States); *L. monocytogenes* detected and quantified according to the ISO method (ISO 11290-1:1996) and *Salmonella* spp. detected and quantified according to the ISO method (ISO 6579-1: 2002). To

confirm the growth of the starters, 5 colonies were collected from MRS agar plates and TSM agar and then identified by the methods reported in Iacumin et al. (2009), briefly: from MRS and TSM agars 5 colonies per plate were isolated and after purification were subjected to Polymerase Chain Reaction (PCR) and the PCR products (V3 16S rRNA amplicons), after purification, were sent to a commercial facility for sequencing (MWG Biotech, Ebersberg, Germany). The sequences were aligned in GenBank using the Blast program version 2.2.18.

2.2.7 Physico-chemical analyses

The control samples (1) and samples inoculated with the starters (7, 8, 9, and 10) alone were subjected to physico-chemical analyses. In particular, the pH was measured at three different points using a pH metre (Basic 20, Crison Instruments, Barcelona, Spain) by inserting the probe directly into the product. The Aw was measured with an Aqua Lab 4 TE (Decagon Devices, Pullman, WA, USA). Humidity was measured according to the AOAC (1990), and NaCl and TVB-N (total volatile basic nitrogen) were measured according to Pearson (1973). Water phase salt was determined according to the formula according to Huss et al. (1997):

$$WPS\ (g|100\ mL) = \frac{salt\ content\ (g|100g)}{moisture\ content\ (mL|100g)\ x\ salt\ content\ (g|100\ g)}\ x\ 100$$

Thiobarbituric acid reactive substances (TBARS) were determined according to Ke et al. (1984). The pH values were detected at 0, 15, 30, 45, and 60 days, while the other physico-chemical parameter at the beginning (0 day) and at the end (60 days) of the shelf-life. At each time point, the analyses were performed in triplicate.

2.2.8 Sensory analysis

Sensory analysis was performed by 20 untrained tasters. Ten additional samples of treatments 1 and 7 were evaluated by tasters who were asked to evaluate the influence of the LAB starter on the organoleptic and sensory characteristics of the products. Sensory analysis was performed based on the triangle test (ISO 4120:2004; triangle test). In short, 20 untrained tasters were presented with three products, two of which were identical. The choice of tasters was mandatory because they represent typical consumers. The tasters were asked whether they understood the examined differences, and in the case of differences they were asked to

specify the type, for example, colour, texture, bouquet, flavour, or smell. Among the samples with starter added, only seven were tested, considering that the LAK-23 was the only strain inhibiting the growth of *L. monocytogenes*.

2.2.9 Statistical analysis

Data were analyzed using Statistica 7.0 version 8 software (StatSoft, Tulsa, OK, USA). The values of the different parameters evaluated were compared by one-way analysis of variance (ANOVA) and the means were then compared using Tukey's honest significance test. Differences were considered significant at $p<0.05$.

2.3 Results

2.3.1 Physico-chemical analyses

Physico-chemical analyses were performed for all the samples. The values of samples with starters added were grouped into two group. The first group includes the samples added with *Carnobacterium*, which showed a not-significant variation in pH during storage ($p>0.05$) (Table 2.1). The second group includes the samples added with *Lacticaseibacillus casei* 211, *Lacticaseibacillus paracasei* 106, and *Latilactibacillus sakei* (LAK-23). In this case, it was observed a slight decrease between 0 and 30 days, and a significant drop between 30 and 60 days ($p < 0.05$). Despite the growth of the added starter, the decrease in pH was approximately 0.1 units.

Table 2.1 pH evolution in cold-smoked sea bass stored at 6 ± 2 °C with and without LAB starter cultures added.

Days	Control	*Carnobacterium*	*L. casei* 211	*L. paracasei* 106	LAK-23
0	5.94 ± 0.05^{a}	5.94 ± 0.05^{a}	5.94 ± 0.05^{a}	5.94 ± 0.05^{a}	5.9 ± 0.05^{a}
15	5.95 ± 0.03^{a}	5.97 ± 0.02^{a}	5.93 ± 0.02^{a}	5.99 ± 0.02^{a}	5.80 ± 0.02^{b}
30	5.96 ± 0.05^{a}	5.96 ± 0.04^{a}	5.91 ± 0.07^{a}	5.86 ± 0.02^{b}	5.90 ± 0.01^{a}
45	5.99 ± 0.05^{a}	5.91 ± 0.06^{a}	5.81 ± 0.03^{b}	5.83 ± 0.03^{b}	5.80 ± 0.07^{b}
60	5.95 ± 0.03^{a}	5.90 ± 0.03^{a}	5.81 ± 0.07^{b}	5.84 ± 0.13^{ab}	5.80 ± 0.08^{b}

Legend: all data are represented as mean ± standard deviation; means with same letters. following the columns, denote not significant differences ($p>0.05$). LAK-23: *Latilactobacillus sakei*.

The values of moisture, NaCl and Aw did not change significantly ($p>0.05$) in the controls and samples inoculated with starter (Table 2.2). The Aw remained within the range of 0.970

and 0.971. In all samples, no significant difference was observed over time ($p > 0.05$). However, it is thought that the small differences in Aw observed at the various sampling points were probably due to variation among the samples and were not correlated to real water loss. The moisture content remained fairly constant over time. It exhibited values between 59.21 and 59.55%, and the observed differences were probably due to the different samples analyzed rather than the absorption or loss of moisture (Table 2.2). The WPS value varied according to the tested samples, but it was over 5.0% in all samples (Table 2.2). These values are acceptable for cold-smoked fish. In this study, the salt and WPS values varied over the storage period without showing a specific trend, indicating that the observed differences were due only to variability among the samples. The salt content was influenced by the variability of the samples and the salting procedure. For these reasons, the observed decreases cannot be considered trends but were due to random heterogeneity in the samples.

Table 2.2 Physico-chemical parameters in cold smoked sea bass stored at 6 ± 2 °C with or without starters added.

Parameters	Days			
	0	0*	60	60*
% Moisture	59.21 ± 0.15[a]	59.51 ± 0.44[a]	59.60 ± 0.31[b]	59.55 ± 0.25[b]
% NaCl	3.3 ± 0.11[a]	3.2 ± 0.60[a]	3.0 ± 0.21[a]	3.1 ± 0.11[a]
Aw	0.970 ± 0.002[a]	0.970 ± 0.001[a]	0.971 ± 0.009[a]	0.971 ± 0.002[a]
% WPS	5.2 ± 0.03[b]	5.0 ± 0.11[a]	5.1 ± 0.5[c]	5.1 ± 0.18[d]
TVB-N mg N/100 g	30.2 ± 0.11[a]	33.05 ± 1.00[b]	35.50 ± 0.28[c]	35.00 ± 0.28[c]
TBARS nmol/g	5.5 ± 0.2[a]	6.1 ± 0.2[b]	6.6 ± 0.3[c]	6.4 ± 0.5[c]

Legend: WPS: Water Salt Phase; TVB-N: Total volatile basic nitrogen; TBARS: Thiobarbituric acid reactive substances - malonaldehyde index. Data represent the means ± standard deviations of the total samples; Means with the same letters, follow the lines and considering each parameters, are not significative different ($p<0.05$).

Additionally, the average values of TVB-N and TBARS (Table 2.2) were largely acceptable. The smoked sea bass showed average values of TVB-N of 30.2 and 33.05 mg N/100 g immediately after packaging, which and increased to 35.50 and 35.00 mg N/100 g at the end of the shelf-life. Indeed, significant increases were observed over time ($p<0.05$), althought limited to 3–5 mg N/100 g. The TBARS increased during storage but remained at the maximum level of 6.6 nmol malonaldehyde/g at the end of storage (60 days). However, over time, an increase in this parameter was observed. At 0 day, the TBARS values were 5.5 and 6.1 nmol malonaldehyde/g, and then they increased significantly, reaching levels of 6.4–6.6 nmol malonaldehyde/g ($p>0.05$).

2.3.2 Microbiological analyses

Lacticaseibacillus casei 211, *Lacticaseibacillus paracasei* 106, *Carnobacterium maltaromaticum*, and *Latilactobacillus sakei* (LAK-23) were the bioprotective cultures used in this work against *L. monocytogenes*. Their use was based on the qualified presumption of safety (QPS), which indicates the safety status of microorganisms intentionally used in food and the feed chain, certifying that they do not pose a risk to human or animal health based on the scientific literature (EFSA BIOHAZ Panel, 2020).

All starter cultures and *L. monocytogenes* grew during storage when they were separately inoculated and reached 7–8 Log CFU/g (data not shown). Additionally, endogenous TBC and LAB (control samples) grew and reached approximately 7 Log CFU/g at the end of sampling (Figures 2.1, 2.2). Consequently, cold-smoked fish represent a good substrate for microorganism growth. Indeed, in the control samples, TBC grew constantly during the entire period, as well as indigenous LAB (Figures 2.1, 2.2).

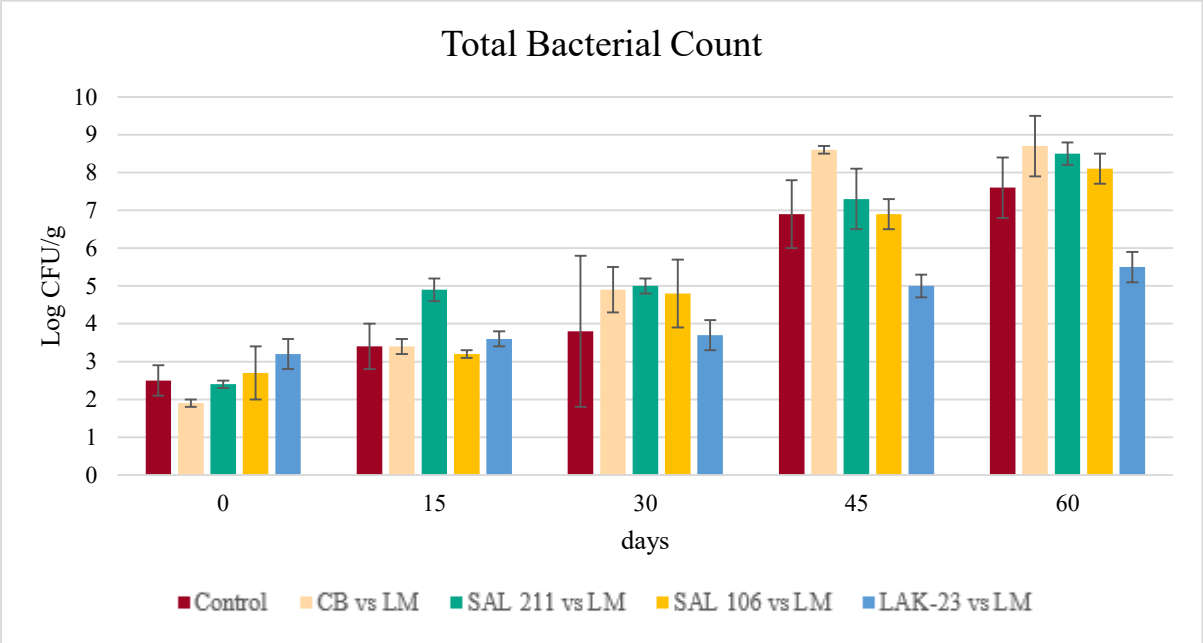

Figure 2.1 Evolution of Total Bacterial Count in cold-smoked sea bass with or without bioprotective starter added, stored at 6 ± 2 °C.

Lactic acid bacteria, usually contaminate substrates and increase in number during the shelf-life of products, they do not present a hazard to consumers, but sometimes, heterofermentative LAB can influence the quality of final products.

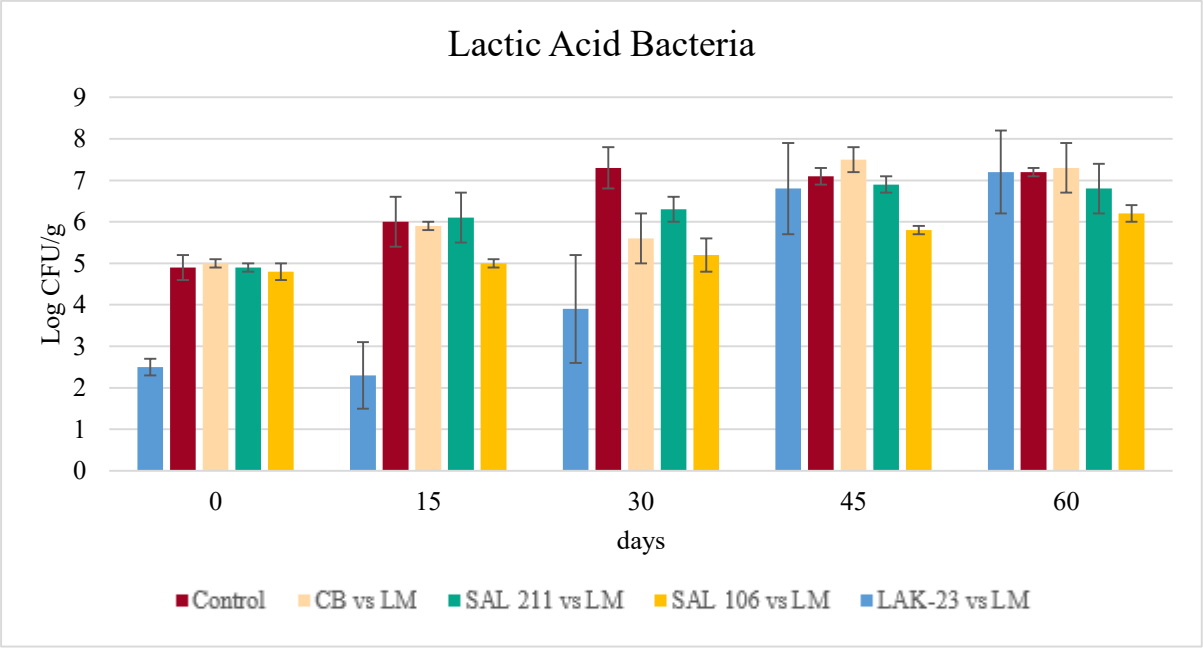

Figure 2.2 Evolution of Lactic Acid Bacteria in cold smoked sea bass with or without bioprotective starter, stored at 6 ± 2 °C.

Other analyses were also performed to confirm that anaerobes and coliforms were not present in the samples. Finally, as expected, in the control samples, the presence of *L. monocytogenes* was below the detection limit (absence in 25 g). In addition, the presence of autochthonous LAB did not influence the growth of *L. monocytogenes*. In samples in which only the pathogen was inoculated, indigenous LAB increased but did not influence the growth of *L. monocytogenes*, which reached high concentrations, as illustrated in Figure 2.3. Cold-smoked sea bass is indeed a suitable substrate for the growth of *Listeria*, despite the temperature of storage, presence of smoking compounds, and high salt concentration (3.5%).

The trends from trials of *Lacticaseibacillus casei* 211, *Lacticaseibacillus paracasei* 106, and *Carnobacterium* spp. coinoculated with *L. monocytogenes* are shown in Figures 2.1–2.3. As shown, TBC and the bioprotective starter grew during the overall storage time. Unfortunately, the coinoculated *L. monocytogenes* also grew, reaching hazardous level (8 Log CFU/g). Consequently, data demonstrated that *Lacticaseibacillus casei* 211, *Lacticaseibacillus paracasei* 106, and *Carnobacterium maltoaromaticum* were not able to limit the growth of the pathogen, which could have become a serious risk to health (Figure 2.3). Figure 2.2 shows the evolution of *Lacticaseibacillus paracasei* 106 coinoculated with *L. monocytogenes*. Even in these trials, TBC evolved over time, increasing from approximately 2.5 Log CFU/g to 8 Log CFU/g (Figure 2.1). *Lacticaseibacillus paracasei* 106 increased significantly from 0 to 60 days, reaching approximately 7 Log CFU/g (Figure 2.2).

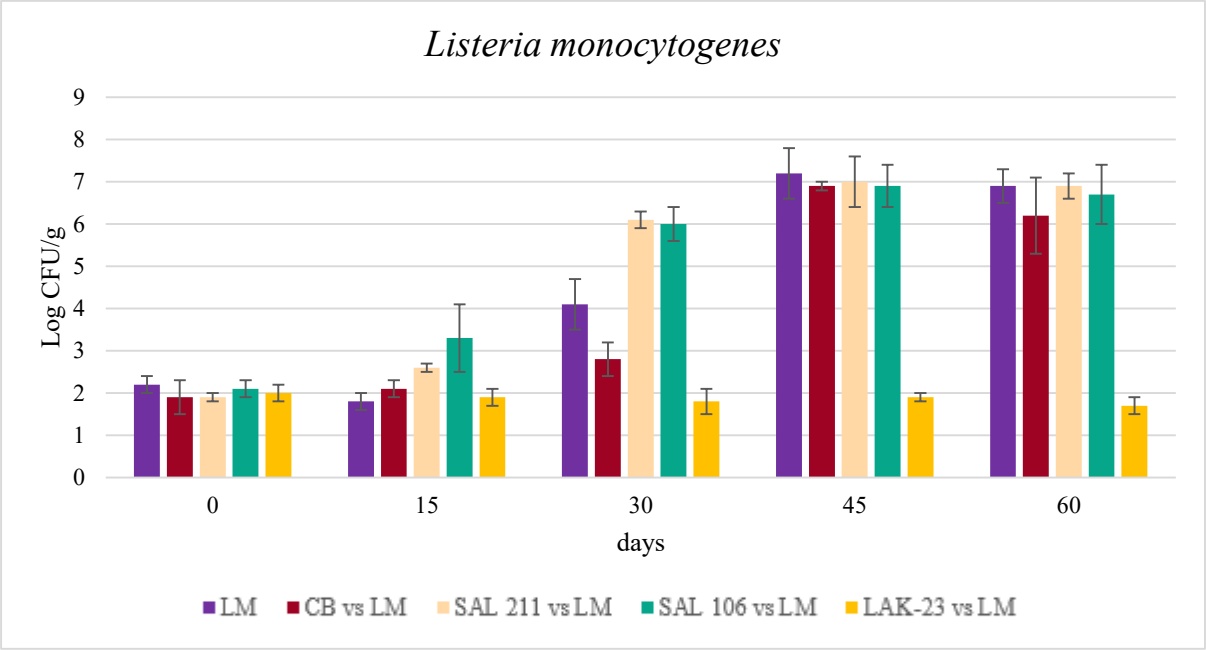

Figure 2.3 Evolution of *Listeria monocytogenes* intentionally inoculated in cold smoked sea bass with or without bioprotective starter, stored at 6 ± 2 °C.

Coinoculated *L. monocytogenes* showed significant increases until 60 days, prevailing over the starter culture through 45 days. Then, at 60 days, its concentration was less than that of *Lacticaseibacillus paracasei* 106 (Figure 2.3). Figures 2.2, 2.3 highlight the evolution of the microbial population of cold-smoked sea bass fillets intentionally inoculated with the starter LAK-23 and *L. monocytogenes*. The starter developed (Figure 2.2) and markedly inhibited the inoculated pathogen *Listeria*. In fact, the concentration of the *L. monocytogenes* mix remained almost constant over time (Figure 2.3), although slight decreases were noted at 45 and 60 days, which they were not significant ($p>0.05$). Therefore, the starter used was effective, and although it did not reduce or completely eliminate the inoculated strains of *L. monocytogenes*, it still prevented their growth. Additionally, TBC grew in samples inoculated with LAK-23 (Figure 2.1), reaching values of 6 Log CFU/g at the end of the shelf-life. Finally, the growth of the added starters in samples inoculated with *L. monocytogenes* was confirmed by the identification of the colonies isolated from MRS and TSM agar (data not shown).

2.3.3 Sensory analysis of cold-smoked sea bass fillets

The samples were brought to environmental temperature immediately before the administration and checked for the presence of atypical odors and flavours, white or viscous patinas, slime, discolouration, or browning. Neither the samples nor the controls were positive

for the previously described parameters and consequently underwent the sensory evaluation. The sensory acceptability of samples with or without starter was determined by the triangular test. No sensory differences were perceived between samples inoculated with LAK-23 and control samples (uninoculated). In fact, neither group of samples was recognized as different. From the comparison of the samples under analysis, it emerged that they belonged to a single sample. Therefore, the starter did not alter the sensory characteristics of the product to which it was added.

2.4 Discussion

The physico-chemical parameters of the cold-smoked sea bass indicated that the moisture and salt concentrations did not change over time, and the minimal variations observed were due to variation among the samples. The pH dropped by 0.1- 0.15 units in samples inoculated with starters, including *L. paracasei* 106, *L. casei* 211 and LAK-23 (*L. sakei*). Lactic acid bacteria produce lactic acid and other acids, which decrease the pH of food (Comi, 2016). The above species used as starter cultures are recognized as able to produce acids, and consequently, their growth can produce a pH decrease. Carnobacteria are known to have a lower acidifying activity compared to other LAB species related to food products (Afzal et al., 2010). This was confirmed also in this study, the pH values of the samples inoculated with *Carnobacterium* spp. have been subjected to a slight variation compared to those of the other samples. Although the pH values of the samples inoculated with LAB were lower compared to those of the control, the decrease was probably neutralized by amino acids produced by proteolysis, biogenic amines and TVB-N. The limited amount of sugar present in these types of products has further limited the production of organic acids by LAB metabolism that is related to the presence of sugar (Cocolin et al., 2006; Urso et al., 2006; Comi, 2016). Moreover, also low storage temperatures can decrease the production of lactic acid, as demonstrated by Petäjä-Kanninen & Puolanne (2007). The values of salt and WPS were adequate for cold-smoked fish. Indeed, regarding the salt content and WPS, the literature underlines the importance of considering a WPS value of 3.5% as the minimum value capable of preventing the growth of *Clostridium botulinum* type E, which is psychotrophic, at the lower storage temperature of 4.4 °C (Centre for Food Safety and Applied Nutrition, 2001). In this study, the salt and WPS

values varied over the storage period without showing specific trends, indicating that the observed differences depended only on variability among the samples and the salting procedure. The observed WPS values, which were closely related to the percentage of salt present, ranged from 4.1 to 5.2%, and these values largely satisfy the limits set by the Centre for Food Safety and Applied Nutrition (2001). These values can ensure health safety throughout the storage time, as demonstrated by different authors, who found WPS values over 4% in Italian and French smoked salmon (Espe et al., 2004; Cornu et al., 2006; Bernardi et al., 2009, 2011). It is likely that the high WPS value may have influenced the growth of CBT and LAB in the tested smoked sea bass samples. In fact, these values are clearly lower than those observed by Bernardi et al. (2009) in smoked salmon. The average values of TVB-N in the samples were largely acceptable, as they were within the standard parameters of smoked seafood, although Chilean authorities (Sernapesca, 1996) have set a limit of 30 mg N/100 g for cold-smoked salmon. Considering the literature data, such a low value seems unattainable (Buchrieser et al., 1993). The smoked sea bass showed average values of TVB-N at the end of storage of 35.50 (samples with starter) and 35.00 mg N/100 g (control). Indeed, a significant increase was observed over time. However, the increase was limited to 3–5 mg N/100 g. The final values of TVB-N were close to 35 mg N/100 g and clearly lower than the maximum values (40 mg N/100 g) proposed by Cantoni et al. (1993) for smoked salmon. In any case, the data regarding TVB-N are different from those obtained by Bernardi et al. (2009), who observed TVB-N values of 49.8 mg N/100 in Italian smoked salmon at the end of shelf-life. It must be noted that the same authors showed initial TVB-N values of approximately 38.2 mg N/100 g, which are values significantly higher than those observed at time 0 in smoked sea bass fillets. Additionally, Leroi et al. (1996, 1998) observed TVB-N values equal to 52.8 mg N/100 g, and these values are significantly higher than those observed here. According to several authors (Ke et al., 1984; Man & Ramadas, 1998), food products are not rancid when TBARS values are <8 nmol malonaldehyde/g product, slightly rancid when TBARS are between 9 and 20 nmol malonaldehyde/g, and rancid and unacceptable when TBARS are >21 nmol malonaldehyde/g. Consequently, all samples of cold-smoked sea bass were considered acceptable, given the low TBARS values. Moreover, the tasters did not perceive hints of rancidity. Cold-smoked seabass is a fishery product that, at the end of production, is usually free from *L. monocytogenes*. However, in the case of contamination in

the production chain, this pathogen can multiply during the product shelf-life. Cold-smoked sea bass shows the ideal growth characteristics for *L. monocytogenes*: a pH higher than 5 units, as shown by the physico-chemical analysis carried out in this study, and an Aw of approximately 0.96, as also reported in a study conducted by Aymerich et al. (2019) in cold-smoked salmon. Even the storage temperature plays a fundamental role. In general, cold-smoked sea bass is a ready-to-eat (RTE) product that needs to be stored constantly at 4°C. However, it is possible that during its shelf-life, the product could face thermal abuse, further contributing to the growth of *L. monocytogenes*. In our study, this condition was tested by keeping the seabass at 6 ± 2°C for the overall period of storage (60 days). In addition to the pH, Aw and temperature, the salt content is also important. Cold-smoked sea bass, as to other similar products, has a salt content of 3.5% (NaCl), but this level is not enough to limit *L. monocytogenes* growth since the pathogen can easily multiply in matrices containing up to 10–12% NaCl. As demonstrated by Vaz-Velho et al. (2005), variation in the salt content of smoked fish samples is unlikely to affect *L. monocytogenes* growth. Therefore, the use of the selected LAB as bioprotective starter cultures presents a valid way to prevent or limit the development of pathogens such as *L. monocytogenes*. *Listeria monocytogenes* was reduced in whole milk by different *Lactobacillus* strains (Garcia et al., 2020) and by *Carnobacterium piscicola* in refrigerated food (Campos et al., 1997). Nevertheless, in this research, the starter cultures acted differently. Two LAB cultures (*L. casei* 211 and *L. paracasei* 106) did not have any effect against *L. monocytogenes* since the pathogen reached high concentrations at 30 days. Indeed, *Carnobacterium maltaromaticum* showed minimal growth control against *L. monocytogenes*, which increased by only 1 Log CFU/g within 30 days. However, the effect was not sufficient when considering the overall period (60 days) in which the pathogen concentration reached hazardous levels (7 Log CFU/g). The failure to compete with *L. monocytogenes* in the long term through the production of bacteriocins could be attributed to many different factors. One could be the presence of food components in the substrate that might affect bacteriocin production and activity (Aasen et al., 2003). In this regard, Dos Reis et al. (2011) considered important the influence of the food matrix on *Carnobacterium*, as the use of natural preservatives in some cases inhibited bacteriocin production, and Vaz-Velho et al. (2005) noticed that bacteriocin activity was reduced, particularly after the smoking process in cold-smoked salmon and trout. In addition, Aymerich et al. (2019) showed that a

Carnobacterium strain exhibited antilisterial activity *in vitro* but did not exert a significant antilisterial effect in all tested products. For this reason, the food matrix and smoking phase could have influenced the ability of the three starters (*L. casei* 211, *L. paracasei* 106 and *Carnobacterium maltaromaticum*) to produce bacteriocins, which could explain why *L. monocytogenes* reached to very high concentrations. On the other hand, LAK-23 (*Latilactobacillus sakei*), was able to inhibit *L. monocytogenes* growth in cold-smoked sea bass. In particular, at the end of storage, *L. monocytogenes* was found at the level of the initial inoculum (2 Log CFU/g). This strain is used either as a starter to promote food ripening or as a bioprotective culture. In particular, considering its fast growth at storage temperatures and bacteriocin production, it was suggested for use in processed meat, and in particular, fish products (Sacco, https://www.saccosystem.com, accession 01/10/2021). Various studies have demonstrated the role of LAB (*Lactobacillus*, *Carnobacterium*, and *Enterococcus*) and bacteriocins in inhibiting *L. monocytogenes* in smoked fish products (Nilsson et al., 1997; Leroi et al., 1998, 2015; Richard et al., 2004; Weiss & Hammes, 2006; Tomé et al., 2008; Concha-Meyer et al., 2011; Rotariu et al., 2014). However, the use of bacteriocins such as nisin and sakacin P was effective against *L. monocytogenes* only in the short term, and consequently, the direct use of bacteriocin-producing starters or the addition of other antimicrobials such as organic acids or essential oils has been suggested (Vaz-Velho et al., 2005; Tomé et al., 2008; Tocmo et al., 2014). Indeed, the application of nisin showed a listeriostatic effect in cold-smoked rainbow trout for only 3 days (Nykänen et al., 2000). However, upon combining nisin and lactate, the *L. monocytogenes* count showed a 2 Log reduction after 17 days of storage (Nykänen et al., 2000). In another study, the use of sakacin P and *L. sakei* Lb790 resulted in a 2 Log reduction in *L. monocytogenes* count after 28 days (Katla et al., 2001). These studies indicate that the potential synergistic effects of combining bacteriocins with other hurdles can extend the duration of inhibition. However, better results have been obtained using bioprotective starters directly, as demonstrated by Aymerich et al. (2019). These authors evaluated three potential bacterial strains against *L. monocytogenes* in smoked salmon with different physico-chemical characteristics (fat concentration, moisture and acetic acid). Among the strains used, two were bacteriocin producers isolated directly from smoked salmon and identified as *Lactobacillus curvatus* and *Carnobacterium maltaromaticum*, while the third was of meat origin and identified as *Lactobacillus sakei*

CTC494, which is a bacteriocin producer. The data demonstrated that *L. sakei* CTC494 inhibited the growth of *L. monocytogenes* after 21 days of storage at 8 °C in all products tested, while *L. curvatus* CTC1742 only limited the growth of the pathogen (<2 Log increase). The efficacy of *C. maltaromaticum* CTC1741 depended on the type of product. In fact, it limited the growth of the pathogen only in one type of smoked salmon. The results obtained by Aymerich et al. (2019) suggest that *L. sakei* CTC494, despite having been isolated from meat, can potentially be used as a bioprotective agent to improve the food safety of cold-smoked salmon. Likewise, *Latilactobacillus sakei* (LAK-23), despite it was isolated from meat products, demonstrated to be able to inhibit *L. monocytogenes* growth in cold-smoked sea bass. The presence of intentionally inoculated selected LAB can also have other positive effects. As illustrated in the microbiological analyses, indigenous LAB could also grow in the product, achieving high concentrations (approximately 7–8 Log CFU/g). Inoculation with selected starter cultures can prevent the development of indigenous microbial populations and, consequently, limit the release of secondary products such as CO_2 and ethanol. These substances are not considered to positive affect the quality of RTE sea bass, as they can cause alteration of sensory characteristics (odor, colour, and texture) and packaging swelling. As cold-smoked sea bass is packed under vacuum, gas production caused the loss of vacuum. Many studies have observed that some organoleptic characteristics are less altered in products treated with LAB compared with untreated samples and that the use of selected starter cultures leads to an improvement in sensory characteristics and antioxidant capacities (Udomsil et al., 2011; Anacarso et al., 2014; Li et al., 2021; Zhou et al., 2021). This is due to LAB competition with endogenous microbiota, such as spoilage bacteria. LAB are also able to produce positive compounds, such as volatiles, and to reduce negative compounds (Huang et al., 2021). These capabilities provide the preservation and improvement of food quality and shelf-life extension. In addition, the utilization of multibacterial mixed fermentation can improve food characteristics and produce different positive compounds than single bacterial fermentation (Huang et al., 2021).

2.5 Conclusions

The LAB contained in the starter grew throughout the storage period and did not inhibit the growth of the pathogen. Only LAK-23 was able to stop the increase in the *L. monocytogenes* load, which remained at the inoculum level until the end of storage. Thus, its use as bioprotective agent is suggested. Therefore, although cold-smoked sea bass does not have a $pH \leq 4.4$ and $Aw \leq 0.92$ or $pH \leq 5.0$ and $Aw \leq 0.94$, as stated in Reg. CE 2073/2005, the results affirm that these products with bioprotective starters inoculated are not a favorable medium for the growth of *L. monocytogenes*. Consequently, they should be included into category 1.3 (ready-to-eat foods that do not constitute a favorable medium for the growth of *L. monocytogenes*, other than those intended for infants and special medical purposes), in which a maximum concentration of *L. monocytogenes* of 100 CFU/g is allowed. In addition, the sensory analysis demonstrated that the use of starters does not depreciate nor mischaracterize cold-smoked sea bass.

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3. Improving the Shelf-Life of Fish Burgers Made with a Mix of Sea Bass and Sea Bream Meat by Bioprotective Cultures

Results here presented were published in:

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3.1 Introduction

Fish are considered a functional food because of their high nutritional value, characterized by presenting components, such as mineral salts, high biological value proteins, and fatty acids (Breda et al., 2017; Samples, 2015). All the components are strictly necessary for the human organism because they are recognized as useful for the prevention of heart and brain diseases (Breda et al., 2017; Nestel et al., 2015). Fish consumption is widespread in Italy, with different consumption amounts from one area to another. In 2021, the consumption of fish was 21 kg/family, but the gap between the different households is significant: an average consumption of 15 kg in families with members under 30 (prefamily and new families), in fact, corresponds to a consumption of over 30 kg in older couples (www.repubblica.it [accessed on 22 August 2022]; www.ismeamercati.it; [accessed on 22 August 2022]). To increase fish consumption, two strategies are necessary: continuous training in school and the transformation of processed fish into derived products. The first strategy is very important because the preferences formed in childhood normally continue into adulthood, and for this reason, at each level of school, meal programs must include fish because they can contribute to the formation of healthy food habits (Breda et al., 2015, Latorres et al., 2016). The second strategy is more important than the first. It is well known that people sometimes give up eating fish due to the presence of fish bones and the characteristic fish odor. Therefore, fish derivatives such as burgers can be a real strategy to increase fish consumption. Indeed, burger production allows the elimination of fish bones and a decrease in the characteristic odor of

fish, which are the main barriers to fish consumption (Belusso et al., 2016), while maintaining the same high nutritional value of the whole fish (Belusso et al., 2016; Mitterer-Daltoé et al., 2014; Corbo et al., 2008). Different works have demonstrated a positive relationship between appearance and positive hedonic perception by consumers with respect to fish derivatives (Latorres et al., 2016; Donadini et al., 2013). Fish are a very highly perishable product with a shelf-life (generally a few days) limited by microbial growth (Iacumin et al., 2021; Corbo et al., 2009). Consequently, fish burgers also have a limited shelf-life. Microorganisms represent the main cause of spoilage, resulting in the formation of nitrogen compounds, sulfides, alcohols, aldehydes, ketones, and organic acids with unpleasant and unacceptable off-flavours (Iacumin et al., 2021; Corbo et al., 2009; Gram & Dalgaard, 2002; Iacumin et al., 2022; Comi, 2017). The short shelf-life of fresh seafood is often because of the growth of specific spoilage organisms (SSOs) (Gram & Dalgaard, 2002; Comi, 2016; Gram & Huss, 1996). The SSO consortium can differ among products (e.g., whole, gutted, and filleted fish) due to a series of factors, such as the composition of the initial microbiota (including the level and type of contamination), type of product, storage conditions, and microbial interactions (Boziaris et al., 2017). Such differences can lead to different shelf life of the products, even when they are stored under the same storage conditions, since different bacterial genera, species, or strains can present different growth rates or metabolism (Syropoulou et al., 2021). Consequently, SSO inhibition by different strategies can improve the microbial quality and prolong the shelf-life of either fish or fish products. Considering the perishability of fish meat, usually to achieve lower rates of spoilage and extend the shelf-life for a long time, mild procedures rather than more drastic means inhibiting SSOs represent useful approaches (Corbo et al., 2009; Comi, 2017; Syropoulou et al., 2021). Among them, modified atmosphere packaging (MAP) or under vacuum packaging, natural preservatives, essential oils (EOs), and, more recently, bioprotective cultures are obtaining success in food research activity as natural compounds with appreciable antimicrobial properties (Iacumin et al., 2021; Corbo et al., 2009; Iacumin et al., 2022; Comi, 2017). Modified atmosphere packaging technology implies the use of several combinations of oxygen, carbon dioxide, and nitrogen, which have different effects on the shelf-life of packaged fish (Poli et al., 2006; Torrieri et al., 2006; Goulas & Kontominas; 2007). The effectiveness of under vacuum packaging depends on the products, the storage temperature, and the experiment (Iacumin et al., 2021, 2022). Data are often in

conflict, and it is difficult to establish which technology between MAP and under vacuum is better (Iacumin et al., 2021; Schelegueda et al., 2016). However, MAP is not always sufficient to preserve processed food and requires combination with other preservation strategies, which are proposed in the literature for seafood products (Olatunde & Benjakul, 2018; Hasani et al., 2020; Dilucia et al., 2021). The most widely used approach for fresh fish burgers is based on the adoption of natural compounds that are properly encapsulated or combined with modified atmospheric conditions (Hasani et al., 2020; Cedola et al., 2017) or enclosed in edible films (Albertos et al., 2019). Among them, EOs exert appreciable antimicrobial properties due to the high content of phenolic derivatives, and they are potentially able to extend the shelf-life of seafood (Corbo et al., 2009; Mahmoud et al., 2007). The use of natural microbiota and/or their antimicrobial products as a biopreservation method is a recent and interesting approach to improve microbial food quality and safety (Iacumin et al., 2022; Danza et al., 2018). Selected LAB strains could be used as bioprotective cultures, as they exert an antagonistic effect against potential pathogens and other undesired microorganisms by the competition for nutrients, pH lowering, and the production of inhibitory compounds such as lactic acid, diacetyl, fatty acids, CO_2, peroxide, and bacteriocins (Danza et al., 2018; Comi & Iacumin, 2017). The antagonistic effect against spoilage or pathogenic microorganisms is obtained either by directly adding living cultures or purified antagonistic substances or fermentation products (Iacumin et al., 2021, 2022; Comi & Iacumin, 2017; Schillinger et al., 1996). The aim of the present study was to develop fish burgers made with a mix of sea bass and sea bream meat and to improve their shelf-life by bioprotective cultures.

3.2 Materials and methods

3.2.1 Bioprotective starter suspension

The commercial starters (BOX-57: *Carnobacterium divergens*, *C. maltaromaticum* and *L. sakei*; LAK-23: *L. sakei*; FP-50: *C. divergens* and *C. maltaromaticum*) were freeze-dried in a foil pouch. F-106 (*Lacticaseibacillus casei*) was derived from the Collection of the Department of Agricultural, Food, Environmental, and Animal Sciences of the University of Udine and was grown in de Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Milan, Italy). After its growth, the strain was harvested by centrifugation at 9700 × *g* for 10 min and then

diluted in saline-peptone water. The commercial starters were thawed, homogenized, and diluted in saline-peptone water (NaCl 0.6%, bacteriological peptone [Oxoid, Milan, Italy] 0.1%, and distilled water) at the time of use. To evaluate the load of each starter, dilutions were performed in saline-peptone water, and 0.1 mL of each dilution was inoculated in the MRS medium by the double layer method for the LAB and in TSM agar (Tryptic Soy medium with 5% glucose, 2% NaCl, and pH 8, Oxoid, Milan, Italy) in a jar prepared for anaerobic reaction with a gas-packing anaerobic system (BBL, Becton Dickinson, Sparks, MD, USA) for the carnobacteria. The plates were incubated at 37 °C for 48–72 h, and the grown colonies were counted. Each suspension contained, on average, approximately 11 Log CFU/g.

3.2.2 Bioprotective starter inoculum

Sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) weighing approximately 474–578 g and 404–440 g, respectively, were headed, gutted, and filleted. Fillets were then minced, mixed, and divided into five equal batches. Each burger was composed of 68% minced fish (50% sea bass and 50% sea bream), 20.5% potato, 5.5% water, 3% rice flour, 2% vegetable fiber, and 1% NaCl. The first batch was formed into patties and directly packaged and used as a control (CTRL). The other batches were inoculated with starter cultures at a final concentration of 10^5 CFU/g of burgers before being formed into patties. Four batches were prepared with each commercial and F-106 starter culture. The burgers were packed in MAP, consisting of 60% N_2 and 40% CO_2, and placed inside rectangular trays made of PET/PE/EVOH/PE: PET (Polyethylenterephtalat/PE (Polyethylene)/EVOH (Ethylene vinyl alcohol)/PE (Polyethylene), ANTIFOG—EVOH (Ethylene vinyl alcohol). The trays were laminated with a top film consisting of APET/PE/EVOH/PE: Amorphous Polyethylenterephtalat/PE (Polyethylene)/EVOH (Ethylene vinyl alcohol)/PE (Polyethylene). The packaged burgers were stored at 4 ± 2 °C for 10 days and then at 8 ± 2 °C for 20 days, according to the challenge test proposed by AFNOR NF V01-003, 2004: hygiene and safety of foodstuffs. Guidelines for the design of an ageing test protocol for the validation of a microbiological lifetime reports for chilled perishable goods that in case where the cold chain is not sufficiently guaranteed, two temperatures must be used: 1/3 of the shelf-life at T1 (4 °C) and 2/3 at T2 (8 °C) (abuse temperatures). Burgers were analyzed at days 0, 6, 12, 18, 24, and 30 in triplicate to test microbial growth, physico-chemical characteristics, and sensory analyses.

3.2.3 Microbiological analyses

Samples were analyzed by traditional microbiological methods to determine the main microbial population. Fifty grams of each sample was transferred into a sterile stomacher bag, and 100 mL of saline-peptone water (NaCl 0.8%, bacteriological peptone [Oxoid, Milan, Italy], 0.1%, distilled water 1 L) was added and mixed for 3 min in a stomacher machine (PBI, Milan, Italy). Further decimal dilutions were made in the same solution, and the following microbiological analyses were performed in duplicate agar plates: (i) Total Bacterial Count (TBC) in Gelysate agar (gelatin sugar-free agar, Oxoid, Milan, Italy) incubated at 30 °C for 48–72 h, (ii) lactic acid bacteria (LAB) on double layer MRS agar (Oxoid, Milan, Italy), incubated at 30 °C up to 5 days, (iii) *Carnobacterium* spp. in TSM agar incubated at 30 °C for 2 days in anaerobiosis, (iv) *Enterobacteriaceae* in Violet Red Bile Glucose agar (VRBG, Oxoid, Milan, Italy), incubated at 37 °C for 48 h; all data are expressed in Log CFU/g product. *Listeria monocytogenes* was detected according to the ISO 11290-1:1996 method (briefly: 25 g product were added to 225 mL of Fraser broth (Oxoid, Italy) incubated at 30 °C for 24 h, then an aliquot of this broth was streaked on Chromocult Listeria Agar according to Ottaviani/Agosti agar (Biolife, Italy), and incubated at 37 °C for 24 h. On this agar, *L. monocytogenes* produce typical blue-green colonies surrounded by an opaque halo), and *Salmonella* spp., according to the ISO 6579-1:2002 method (briefly: 25 g product were added to 225 mL of Buffered Peptone Water (BPW, Oxoid, Milan, Italy) incubated 18 h at 37 °C, then 1 mL of BPW in 9 mL of Rappaport Vassiliadis broth (RV, Oxoid, Italy) incubated at 42 °C for 18–24 h. An aliquot of RV was streaked on Xylose Lysine Tergitol 4 agar (Oxoid, Italy) incubated at 37 °C for 24 h. On this agar, the black or center black colonies were presumptive *Salmonella*). To confirm the growth of the starters, 5 colonies were collected from MRS agar plates and TSM agar and then identified by the method reported in Iacumin et al. (2009), briefly: from MRS and TSM agars 5 colonies per plate were isolated and after purification were subjected to Polymerase Chain Reaction (PCR) and the PCR products (V3 16S rRNA amplicons), after purification, were sent to a commercial facility for sequencing (MWG Biotech, Ebersberg, Germany). The sequences were aligned in GenBank using the Blast program version 2.2.18.

3.2.4 Physico-chemical analyses

The pH was detected at 3 different points using a pH meter (Basic 20, Crison Instruments, Spain) by inserting the probe directly into the product. The water activity (Aw) was measured with an Aqua Lab 4 TE (Decagon Devices, Pullman, WA, USA), and TVB-N (total volatile basic nitrogen) was measured according to Pearson (1993). To evaluate the oxidation stability during storage, the thiobarbituric acid–reactive substances (TBARS) were determined in triplicate (Ke et al., 1984) (briefly: the Total Volatile Basic Nitrogen (TVB-N) was estimated by boiling a mix of distilled water [50 mL] and 10 g of product in presence of MgO (25 mL, 2% w/v). The distillate was collected in a solution of boric acid and titrated with sulfuric acid in the presence of methyl red. Data are expressed in mg Nitrogen/100 g. The thiobarbituric acid value (TBARS) was determined directly by spectrophotometric quantification of compounds obtained by the distillation of a mix consisting of distilled water (50 mL) and fish product (10 g), acidified with hydrochloric acid (2.5 mL, 4 N) until pH 1.5. Then, 5 mL of the distillate was treated with 5 mL of a solution of thiobarbituric acid (TBA), obtained by mixing TBA in acetic acid (90%), and placed in boiled water for 35 min. After cooling, the solution was read at 538 nm. Three analyses were performed at each sampling point and data are expressed in nmol malonaldehyde/g.

3.2.5 Analysis of volatile compounds (volatilome)

Volatile organic compounds of samples were analyzed with gas chromatography-mass spectrometry coupled with solid-phase microextraction (SPME-GC–MS) using an Agilent Hewlett–Packard 6890 GC gas chromatograph and a 5970 MSD MS detector (Hewlett–Packard, Geneva, Switzerland) equipped with a Varian (50 m × 0.32 mm × 1.2 μm) fused silica capillary column. Samples (3 g) were placed in 10 mL sterilized vials, added to a known amount of 4-methyl-2-pentanol (Sigma-Aldrich, Steinheim, Germany) as an internal standard, and sealed by PTFE/silicon septa. The samples were heated for 10 min at 45 °C and then a fused silica SPME fiber covered with 85 μm Carboxen/Polydimethylsiloxane (CAR/PDMS) (Supelco, Steinheim, Germany) was introduced into the headspace for 40 min. Adsorbed molecules were desorbed in the gas chromatograph for 10 min. The conditions were the same as those reported by Montanari et al. (2018). Volatile peak identification was carried out by computer matching of mass spectral data with those of compounds contained in the libraries NIST 2005 and 2011. Data reported are means of three different burgers.

3.2.6 Sensory analysis

The sensory evaluation panel consisted of 12 untrained assessors. The cooked burgers were presented on white plates at room temperature. Ten burgers of the control and of each treatment were evaluated. Assessors were asked to evaluate the following descriptors: odor (fermentation, rancid, or fishy), taste (sweet, sour, pungent, or rancid), flavour (ammonia, sweet, sour, or bitter) and appearance (slime). The 12 assessors evaluated the presence or the absence of each of the nine descriptors. The results stated for each sample is the sum of the assessors who considered the presence of the descriptor out of the total of the assessors (Baublis et al., 2006; Vàlkovà et al., 2007). Then, the final score is calculated by asking the panelists to give a general evaluation of the sensory quality of the products, within a scale from 1 (excellent) to 5 (worst).

3.2.7 Statistical analysis

Statistical elaboration was carried out by the specific software Statistica for Windows, version 8.0 (StatSoft, Tulsa, OK, USA). Means and standard deviations were calculated, and data were elaborated by principal component analysis (PCA) and by factorial ANOVA (two factors: starter culture and time) and Tukey's HSD test. Significant differences among samples were calculated at $p<0.05$.

3.3 Results

3.3.1 Microbial and physico-chemical characteristics

To evaluate the capability of the different starter cultures to compete with the autochthonous microbial flora of the burgers, viable counts were performed during storage. The results of viable counts of total bacterial count (TBC), *Enterobacteriaceae*, and LAB are reported in Table 3.1. In all samples, TBC increased until 12 days of storage, reaching values between 5.32 and 6.47 Log CFU/g, and then decreased; at the end, it was between 3.01 and 3.58 Log CFU/g. At the beginning of storage, the LAB counts of the inoculated samples ranged between 5.09 and 5.68 Log CFU/g, corresponding to the added amount of starter, and the control samples had 3.42 Log CFU/g. Lactic acid bacteria counts increased rapidly during the first 12 days and reached more than 7 Log CFU/g in all the samples. Thereafter, the growth continued, and at the end of the storage, the counts in the various samples reached maxima,

which ranged from 8.62 to 9.18 Log CFU/g. In all the samples, *Enterobacteriaceae* population increased progressively until day 12, after which it decreased. No significant differences in counts ($p>0.05$) of *Enterobacteriaceae* between the control and inoculated samples were recorded from day 0 to day 18 of storage. Therefore, burgers inoculated with LAK-23 presented low population amounts compared to the other samples. *Listeria monocytogenes* and *Salmonella* spp. were not detected in any of the samples. All pH profiles demonstrated clear acidification as a function of time, both in the control and when a starter culture was added. The initial pH of the burgers ranged from 6.17 to 6.31. On day 12 of storage, the pH values decreased in all the samples, but the acidification in the inoculated samples was higher (Table 3.1). In fact, the pH of the control samples was 5.56, which was significantly higher ($p<0.05$) than that of the inoculated samples, which had pH values between 4.67 and 5.09. In contrast, at day 18, the control, together with FP-50, had lower pH values. Subsequently, the pH values of all the samples remained practically unchanged until the end of storage (4.31–4.45). The Aw of the burgers at day 0 was 0.9836. Total volatile basic nitrogen (TVB-N) is known as a product of bacterial spoilage and endogenous enzyme action, and its level is often used as an index to evaluate fish quality. The levels of these compounds, which increase with the onset of microbial spoilage, are primarily responsible for the fishy odors, which increase as spoilage proceeds (Table 3.1). At the onset of storage, the TVB-N value of burgers was 25.60 mg N/100 g. TVB-N increased with storage time. Significant differences ($p < 0.05$) between the samples were observed from day 18. The increase in TVB-N values was lower in the samples inoculated with FP-50 starter culture. At the end of storage (30 days), the control samples had a higher value of TVB-N. The TVB-N content of all the samples exceeded the maximum level for acceptability for marine fish (i.e., 35 mg/100 g; Reg. CE 2074/2005) at day 12. The pouches were examined daily for swelling during storage. A large portion of the uninoculated burgers (40%) started swelling at 12 days. In contrast, the inoculated burgers did not become swollen during the overall storage period (Figure 3.1). Principal component analysis (PCA) (data not shown) was performed to determine the relationship between design variables (time of storage and starter cultures) and microbial and physico-chemical variables. The samples on the same day of storage were grouped together, regardless of the starter cultures used. As expected, storage had a significant effect on the microbial population and physico-chemical properties of the burgers.

Table 3.1 Fate of physico-chemical and microbial characteristics of burgers made with a mix of sea bass and sea bream meat.

		Days					
	Starter	**0**	**6**	**12**	**18**	**24**	**30**
Total bacterial count (Log CFU/g)	CTRL	4.76±0.12^{a}	4.89±0.47^{a}	6.47±0.63^{b}	3.58±0.17^{a}	3.68±0.67^{a}	3.26±0.19^{a}
	LAK-23	4.71±0.08^{a}	5.00±0.59^{a}	6.03±0.37^{ab}	4.06±0.77^{a}	3.20±0.36^{a}	3.49±0.20^{a}
	F-106	4.89±0.09^{a}	4.73±0.06^{a}	6.33±0.13^{b}	4.33±0.49^{a}	3.94±0.44^{a}	3.58±0.07^{a}
	FP-50	4.85±0.09^{a}	5.19±0.44^{a}	5.32±0.20^{a}	4.00±0.67^{a}	3.01±0.05^{a}	3.27±0.43^{a}
	BOX-57	5.22±0.20^{b}	5.80±0.18^{a}	5.70±0.17^{ab}	3.78±0.30^{a}	3.00±0.17^{a}	3.01±0.15^{a}
Lactic acid bacteria (Log CFU/g)	CTRL	3.42±0.14^{a}	4.44±1.34^{a}	7.55±0.16^{a}	8.73±1.15^{a}	7.83±0.22^{a}	9.18±0.11^{b}
	LAK-23	5.13±0.16^{b}	6.94±0.90^{b}	8.79±0.07^{b}	8.58±0.36^{a}	9.15±0.37^{b}	9.07±0.04^{b}
	F-106	5.09±0.09^{b}	5.90±0.89^{ab}	8.17±0.15^{ab}	8.98±0.21^{a}	8.38±0.23^{ab}	8.62±0.08^{a}
	FP-50	5.68±0.15^{c}	5.64±0.27^{ab}	7.98±0.66^{ab}	9.48±0.79^{a}	8.76±0.67^{ab}	9.11±0.16^{b}
	BOX-57	5.39±0.08^{bc}	6.35±0.86^{ab}	8.36±0.22^{ab}	9.01±0.17^{a}	8.77±0.26^{ab}	8.99±0.16^{b}
***Enterobacteriaceae* (Log CFU/g)**	CTRL	2.74±0.14^{a}	4.67±0.27^{a}	4.58±1.02^{a}	4.01±0.27^{a}	2.39±0.41^{bc}	1.44±0.36^{b}
	LAK-23	2.83±0.18^{a}	4.40±0.71^{a}	5.45±0.32^{a}	3.55±0.47^{a}	0.52±0.15^{a}	0.48±0.01^{a}
	F-106	2.58±0.12^{a}	3.55±1.55^{a}	5.26±1.13^{a}	3.62±0.32^{a}	2.34±0.38^{bc}	3.00±0.30^{c}
	FP-50	2.63±0.29^{a}	4.31±0.22^{a}	3.97±0.11^{a}	4.08±0.59^{a}	1.48±0.00^{b}	2.40±0.09^{c}
	BOX-57	2.75±0.06^{a}	4.48±0.27^{a}	4.27±0.77^{a}	3.66±0.37^{a}	2.61±0.62^{c}	1.20±0.22^{b}
pH	CTRL	6.23±0.03^{ab}	6.25±0.06^{a}	5.56±0.34^{b}	4.36±0.03^{a}	4.30±0.04^{a}	4.31±0.00^{a}
	LAK-23	6.31±0.03^{b}	6.30±0.11^{a}	4.89±0.01^{a}	4.48±0.02^{b}	4.32±0.08^{a}	4.34±0.07^{ab}
	F-106	6.27±0.03^{ab}	6.31±0.06^{a}	5.09±0.14^{a}	4.52±0.09^{b}	4.39±0.05^{a}	4.38±0.02^{ab}
	FP-50	6.29±0.05^{b}	5.99±0.15^{a}	4.67±0.01^{a}	4.36±0.01^{a}	4.23±0.06^{a}	4.37±0.06^{ab}
	BOX-57	6.17±0.04^{a}	6.00±0.19^{a}	4.83±0.05^{a}	4.57±0.02^{b}	4.40±0.07^{a}	4.45±0.05^{b}
TVB-N (mg N/100g)	CTRL	25.60±3.33^{a}	32.80±2.75^{a}	40.03±1.46^{a}	47.27±0.80^{b}	60.93±1.60^{b}	88.63±0.96^{d}
	LAK-23	25.60±3.33^{a}	31.33±2.91^{a}	38.87±1.70^{a}	45.23±2.58^{a}	55.13±1.10^{a}	74.37±0.81^{b}
	F-106	25.60±3.33^{a}	33.00±2.95^{a}	40.40±1.13^{a}	47.80±0.60^{b}	63.50±2.69^{c}	69.07±1.25^{a}
	FP-50	25.60±3.33^{a}	31.40±0.96^{a}	39.73±1.39^{a}	42.50±1.32^{a}	54.63±2.01^{a}	70.50±1.11^{a}
	BOX-57	25.60±3.33^{a}	32.73±3.49^{a}	39.43±0.61^{a}	45.63±0.68^{a}	56.37±1.59^{a}	80.53±0.70^{c}

Legend: CTRL: control, non inoculated; Starter added (BOX-57: *C. divergens*, *C. maltaromaticum* and *L. sakei*; LAK-23: *L. sakei*; FP-50: *C. divergens* and *C. maltaromaticum*; F-106; *Lacticaseibacillus casei*)**.**
Data mean ± standard deviation; Mean with different letters within each day and each character (following the columns) are significative different ($p<0.05$).

The TBARS content changed slightly during storage. However, at time 0 and at 30 days of storage, no significant differences were observed in any of the samples regardless of the treatment (with or without starters added). At 0 days, the TBARS means were at the level of 1.2 ± 0.3 nmol malondialdehyde/g in all the burgers, and then they slightly increased, reaching acceptable levels at 30 days (2.1± 0.5 nmol malondialdehyde/g).

3.3.2 Volatile compound characteristics (VOCs)

Table 3.2 provides the data concerning the volatile molecules detected. The compounds are grouped according to their chemical structure, into aldehydes, ketones, alcohols, acids, and esters. The results are expressed as the ratio between the peak area of each molecule and the peak area of the standard (4-methyl-2-pentanol). Alcohols, and in particular 1-propanol, isopropyl alcohol, and 1-penten-3-ol, were detected in higher amounts in the control at time 0. The second group was represented by aldehydes, among which hexanal, nonanal, and pentanal were the major contributors. All these aliphatic aldehydes derive mainly from lipid oxidation; they are responsible for "green notes", but at higher concentrations, they can result in rancid perception (Ordónez et al., 1999). After 6 days, aldehydes demonstrated a small decrease in the control and in the fish burgers containing the culture F-106, while an increase was observed with BOX-57, FP-50, and LAK-23 samples. Alcohols were characterized by relevant increases, especially in the samples containing bioprotective cultures. These latter were also responsible for high concentrations of ketones, such as diacetyl and acetoin, especially in BOX-57, FP-50, and LAK-23. The same three bioprotective cultures were characterized by a higher accumulation of acetic acid, while the culture F-106 seems to confirm its slower metabolism under the conditions considered here. After 12 days, all the samples containing bioprotective cultures demonstrated higher amounts of aldehydes (in particular, hexanal) than the control, while ketones were quite similar, except for F-106, which presented a relevant accumulation of diacetyl and methyl isobutyl ketone. Acetic acid was extremely low in the control compared with samples supplemented with bioprotective cultures, among which BOX-57 was the maximum producer and F-106 was characterized by a lower amount of this acid, confirming a slower metabolism of this strain in fish burgers. Alcohols increased, especially in the control and in the samples supplemented with F-106 and BOX-57. In addition to ethanol, the alcohol increase in the fish burgers containing bioprotective cultures was due to hexanol (derived from hexanal reduction) and 1-octen-3-ol (which derives from the oxidative degradation of linoleic acid). The samples analyzed at 18 days were characterized by a drastic reduction in the proportion of aldehydes, mainly due to the decrease in hexanal. Additionally, ketones markedly decreased in the samples containing bioprotective cultures. In particular, in all these samples, the major decreases were observed for methyl isobutyl ketone, diacetyl, and acetoin. In contrast, this chemical group increased

in the control, in which great proportions of diacetyl, acetoin, and 2-butanone were detected. The control was also characterized by a relevant increase in alcohol concentration, in particular attributable to the high proportion of ethanol and 3-methyl-1-butanol. Among acids, acetic acid was again the most important molecule, whose amount, despite an increase in the control, remained remarkably higher in the samples containing bioprotective cultures. Ethyl acetate was present in a higher proportion in the control. Considering the deep changes in the volatilome, which greatly influenced the sensorial aspects and the reaching of a high value of TVB-N, it was suggested to stop the VOC analysis at 18 days. To highlight the modification of the volatile profile during storage, Figure 3.2 reports the results of a PCA based on the proportion of the volatile molecules detected in the samples. Factor 1 explains 27.43% and Factor 2 explains 23.45% of the variability. The different samples were grouped according to the sampling time, and within each sampling time, the control was separated from the samples containing bioprotective cultures, among which BOX-57 seems to present the most peculiar profile.

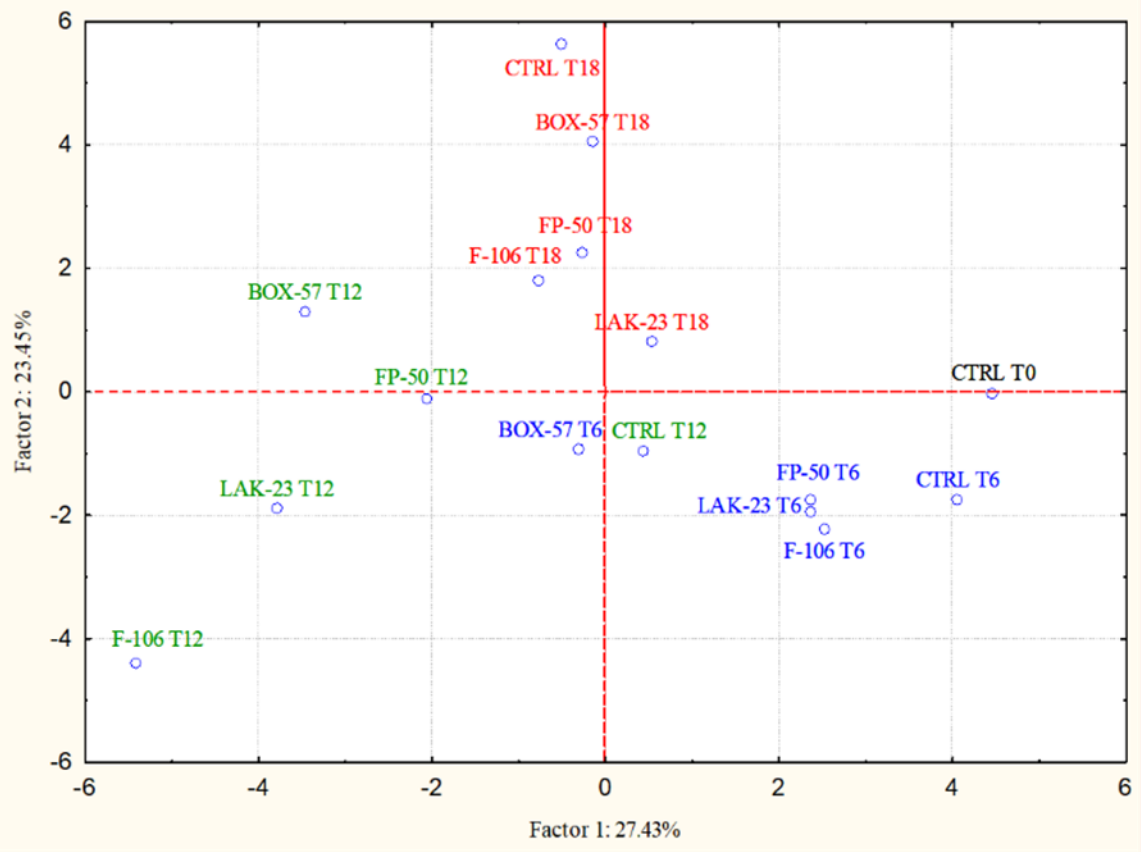
Figure 3.1 Pictures of the uninoculated (A) and inoculated (B) fish burgers at 12 days of storage.

Table 3.2 Mean values of volatile compounds identified at 0, 6, 12 and 18 days of storage in the different burgers.

Volatile compounds	0 days	6 days					12 days					18 days				
	CTRL	CTRL	F-106	BOX-57	FP-50	LAK-23	CTRL	F-106	BOX-57	FP-50	LAK-23	CTRL	F-106	BOX-57	FP-50	LAK-23
Pentanal	1.30	1.87 ^{a}	1.76 ^{a}	2.29 ^{b}	1.91 ^{a}	2.31 ^{b}	1.52 ^{a}	3.97 ^{b}	2.69 ^{c}	2.11 ^{c}	2.65 ^{c}	0.48 ^{a}	1.06 ^{b}	1.42 ^{c}	1.42 ^{c}	1.26 ^{b}
Hexanal	7.28	2.48 ^{a}	1.97 ^{a}	8.89 ^{b}	7.09 ^{c}	9.52 ^{b}	4.36 ^{a}	10.85 ^{b}	5.82 ^{c}	9.52 ^{b}	12.87 ^{b}	2.16 ^{a}	0.43 ^{b}	1.02 ^{c}	2.15 ^{a}	4.73 ^{d}
Nonanal	2.09	2.12 ^{a}	1.73 ^{b}	2.24 ^{a}	1.22 ^{c}	1.83 ^{ab}	1.95 ^{a}	2.43 ^{b}	2.31 ^{ab}	2.17 ^{a}	2.47 ^{b}	1.98 ^{a}	2.38 ^{b}	1.07 ^{c}	1.00 ^{c}	1.23 ^{c}
Benzaldehyde	0.93	2.31 ^{a}	1.97 ^{a}	3.47 ^{b}	2.38 ^{a}	2.69 ^{ac}	1.76 ^{a}	3.04 ^{b}	3.00 ^{b}	2.13 ^{a}	2.06 ^{a}	1.57 ^{a}	2.50 ^{b}	1.95 ^{a}	1.44 ^{a}	2.42 ^{b}
ALDEHYDES	**11.60**	**8.79** ^{a}	**7.42** ^{a}	**16.90** ^{b}	**12.59** ^{c}	**16.35** ^{b}	**9.59** ^{a}	**20.29** ^{b}	**13.82** ^{c}	**15.93** ^{d}	**20.05** ^{b}	**6.20** ^{a}	**6.37** ^{a}	**5.46** ^{ab}	**6.01** ^{a}	**9.64** ^{c}
Acetone	2.46	2.35	2.89	2.48	2.18	2.63	3.46 ^{a}	5.29 ^{b}	3.51 ^{a}	3.00 ^{a}	4.31 ^{c}	1.20 ^{a}	2.63 ^{b}	2.94 ^{b}	3.30 ^{c}	3.63 ^{c}
2-butanone	0.84	0.41 ^{a}	1.09 ^{b}	1.07 ^{b}	0.68 ^{a}	0.75 ^{a}	1.97 ^{a}	2.58 ^{b}	1.72 ^{a}	1.47 ^{a}	1.88 ^{a}	16.68 ^{a}	1.38 ^{b}	1.50 ^{b}	1.59 ^{b}	1.60 ^{b}
Diacetyl	0.00	0.00 ^{a}	0.28 ^{b}	4.97 ^{c}	6.91 ^{d}	2.54 ^{e}	1.93 ^{a}	6.24 ^{b}	3.22 ^{c}	2.00 ^{a}	3.92 ^{c}	4.21 ^{a}	0.30 ^{b}	0.00 ^{b}	0.00 ^{b}	0.90 ^{c}
Methyl isobutyl ketone	3.86	3.18 ^{a}	4.77 ^{b}	3.53 ^{a}	4.51 ^{b}	4.01 ^{bc}	12.81 ^{a}	19.30 ^{b}	8.84 ^{c}	11.20 ^{d}	10.76 ^{a}	6.36 ^{a}	6.38 ^{a}	6.74 ^{a}	7.58 ^{b}	7.34 ^{b}
4-methyl,3-penten-2-one	1.74	1.60 ^{a}	1.36 ^{ab}	1.63 ^{a}	1.80 ^{a}	2.08 ^{a}	0.78 ^{a}	1.12 ^{a}	1.66 ^{b}	1.12 ^{a}	0.91 ^{a}	1.04 ^{a}	1.21 ^{a}	1.34 ^{a}	1.00 ^{a}	2.43 ^{b}
Acetoin	0.00	0.00 ^{a}	1.66 ^{b}	12.18 ^{c}	16.99 ^{d}	8.03 ^{e}	0.00 ^{a}	3.82 ^{b}	4.11 ^{c}	2.88 ^{d}	2.79 ^{d}	30.57 ^{a}	1.12 ^{b}	1.97 ^{c}	1.05 ^{b}	1.89 ^{c}
KETONES	**8.90**	**7.55** ^{a}	**12.05** ^{b}	**25.86** ^{c}	**33.07** ^{d}	**20.05** ^{e}	**20.95** ^{a}	**38.34** ^{b}	**23.06** ^{ac}	**21.68** ^{a}	**24.57** ^{c}	**60.06** ^{a}	**13.01** ^{b}	**14.49** ^{c}	**14.51** ^{c}	**17.78** ^{e}
Isopropyl alcohol	3.19	2.25 ^{a}	4.53 ^{b}	2.39 ^{a}	3.70 ^{c}	2.61 ^{a}	5.27 ^{a}	6.04 ^{b}	3.75 ^{c}	3.62 ^{c}	5.33 ^{a}	3.00 ^{a}	4.49 ^{b}	2.25 ^{c}	3.43 ^{a}	3.15 ^{a}
Ethyl alcohol	0.57	13.68 ^{a}	12.87 ^{a}	24.33 ^{b}	15.42 ^{c}	5.90 ^{d}	20.22 ^{a}	20.16 ^{a}	27.05 ^{b}	16.48 ^{c}	6.21 ^{d}	62.36 ^{a}	15.37 ^{b}	20.74 ^{c}	12.87 ^{d}	9.75 ^{e}
1-propanol	10.92	9.13 ^{a}	10.16 ^{a}	8.45 ^{b}	8.84 ^{ab}	10.59 ^{ac}	14.40 ^{a}	11.90 ^{b}	10.92 ^{b}	9.31 ^{c}	16.19 ^{d}	11.17 ^{a}	8.10 ^{b}	8.37 ^{b}	9.56 ^{c}	10.97 ^{a}
1-penten-3-ol	2.90	3.78 ^{a}	5.37 ^{b}	4.14 ^{a}	4.49 ^{c}	4.45 ^{c}	4.39 ^{a}	7.54 ^{b}	3.74 ^{c}	4.24 ^{a}	5.23 ^{d}	3.36 ^{a}	3.69 ^{a}	2.55 ^{b}	3.32 ^{a}	3.71 ^{a}
1-butanol, 3-methyl	0.00	0.00 ^{a}	0.00 ^{a}	2.91 ^{b}	0.83 ^{c}	0.63 ^{c}	1.25 ^{a}	1.35 ^{a}	3.39 ^{b}	1.61 ^{a}	1.42 ^{a}	11.44 ^{a}	0.91 ^{b}	3.07 ^{c}	1.14 ^{b}	0.72 ^{b}
1-pentanol	1.28	3.65 ^{a}	3.69 ^{a}	4.67 ^{b}	3.24 ^{a}	2.89 ^{c}	3.05 ^{a}	3.60 ^{b}	2.52 ^{c}	3.76 ^{b}	4.05 ^{d}	2.45 ^{a}	1.84 ^{b}	1.80 ^{b}	2.60 ^{a}	2.72 ^{a}
3-buten-1-ol,3-methyl	0.00	0.00 ^{a}	0.00 ^{a}	0.53 ^{b}	0.17 ^{ab}	0.00 ^{ab}	0.33 ^{a}	0.72 ^{b}	0.54 ^{a}	0.93 ^{b}	0.88 ^{b}	0.25 ^{a}	0.67 ^{b}	0.70 ^{b}	0.96 ^{b}	0.91 ^{b}
cis 2-penten-1-ol	1.71	3.26 ^{a}	3.63 ^{a}	3.17 ^{a}	3.15 ^{a}	2.87 ^{b}	1.79 ^{a}	3.03 ^{b}	1.64 ^{a}	2.27 ^{c}	1.78 ^{a}	1.66 ^{a}	1.85 ^{a}	1.13 ^{b}	1.50 ^{a}	1.62 ^{a}
2-buten-1-ol, 3-methyl	0.00	0.00 ^{a}	0.00 ^{a}	0.54 ^{b}	0.25 ^{b}	0.69 ^{b}	1.15 ^{a}	2.21 ^{b}	4.00 ^{c}	3.06 ^{d}	2.98 ^{d}	0.00 ^{a}	5.80 ^{b}	4.19 ^{c}	4.23 ^{c}	3.28 ^{d}
Hexanol	1.94	2.10 ^{a}	2.81 ^{b}	5.39 ^{c}	3.61 ^{d}	4.11 ^{d}	2.47 ^{a}	5.10 ^{b}	6.19 ^{c}	4.46 ^{d}	4.36 ^{d}	4.70 ^{a}	3.41 ^{b}	4.24 ^{b}	3.63 ^{b}	3.03 ^{bd}
1-octen-3-ol	2.78	5.48	5.59	5.49	5.54	5.21	3.92 ^{a}	7.47 ^{b}	4.21 ^{ac}	4.45 ^{c}	4.35 ^{c}	4.51 ^{a}	4.04 ^{a}	2.95 ^{b}	2.85 ^{b}	3.05 ^{b}
Heptanol	1.12	1.38 ^{a}	0.39 ^{b}	0.38 ^{b}	1.09 ^{a}	1.19 ^{a}	0.34 ^{a}	0.00 ^{b}	0.21 ^{a}	0.00 ^{b}	0.00 ^{b}	0.00	0.00	0.00	0.00	0.00
ALCOHOLS	**26.41**	**44.71** ^{a}	**49.03** ^{b}	**62.39** ^{c}	**50.34** ^{d}	**41.14** ^{a}	**58.57** ^{a}	**69.13** ^{b}	**68.17** ^{b}	**54.19** ^{a}	**52.80** ^{ac}	**104.90** ^{a}	**50.17** ^{b}	**51.98** ^{b}	**46.07** ^{b}	**42.94** ^{bc}
Acetic acid	1.12	0.71 ^{a}	1.24 ^{b}	15.57 ^{c}	3.15 ^{d}	5.32 ^{e}	2.96 ^{a}	24.71 ^{b}	66.15 ^{c}	38.58 ^{d}	38.87 ^{d}	16.21 ^{a}	53.03 ^{b}	52.55 ^{b}	48.10 ^{c}	39.48 ^{d}

Propanoic acid	0.00	0.26 ^{a}	0.00 ^{b}	0.70 ^{a}	0.40 ^{a}	0.55 ^{a}	0.46 ^{a}	0.94 ^{a}	1.26 ^{ab}	0.95 ^{a}	1.39 ^{b}	3.49 ^{a}	0.58 ^{b}	0.75 ^{b}	0.64 ^{b}	0.73 ^{b}
Butanoic acid	0.00	0.00 ^{a}	0.00 ^{a}	0.46 ^{b}	0.28 ^{b}	0.40 ^{b}	0.31 ^{a}	0.90 ^{b}	1.62 ^{c}	1.24 ^{b}	1.46 ^{bc}	0.23 ^{a}	1.12 ^{b}	1.03 ^{b}	0.97 ^{b}	0.75 ^{b}
Butanoic acid, 3-methyl	0.00	0.00 ^{a}	0.00 ^{a}	0.74 ^{b}	0.00 ^{a}	0.19 ^{ab}	0.00 ^{a}	0.00 ^{a}	0.74 ^{b}	0.24 ^{ab}	0.00 ^{a}	0.67 ^{a}	0.36 ^{a}	1.26 ^{b}	0.32 ^{a}	0.00 ^{c}
Hexanoic acid	1.11	0.61 ^{a}	0.38 ^{a}	1.85 ^{b}	0.78 ^{a}	0.97 ^{a}	0.70 ^{a}	2.21 ^{b}	2.60 ^{b}	2.19 ^{b}	2.41 ^{b}	0.95 ^{a}	2.56 ^{b}	2.16 ^{b}	1.74 ^{c}	1.39 ^{a}
ACIDS	**2.23**	**1.57** ^{a}	**1.62** ^{a}	**19.32** ^{b}	**4.61** ^{c}	**7.42** ^{d}	**4.43** ^{a}	**28.75** ^{b}	**72.36** ^{c}	**43.20** ^{d}	**44.13** ^{d}	**21.56** ^{a}	**57.65** ^{b}	**57.75** ^{b}	**51.77** ^{c}	**42.35** ^{d}
Ethyl acetate	1.08	3.93 ^{a}	6.83 ^{b}	5.99 ^{c}	4.05 ^{d}	2.50 ^{e}	5.81 ^{a}	14.15 ^{b}	10.44 ^{c}	6.91 ^{d}	5.35 ^{a}	13.99 ^{a}	5.13 ^{b}	5.89 ^{b}	7.00 ^{c}	4.88 ^{bd}
ESTERS	**1.08**	**3.93** ^{a}	**6.83** ^{b}	**5.99** ^{c}	**4.05** ^{d}	**2.50** ^{e}	**5.81** ^{a}	**14.15** ^{b}	**10.44** ^{c}	**6.91** ^{d}	**5.35** ^{a}	13.99 ^{a}	5.13 ^{b}	5.89 ^{b}	7.00 ^{c}	4.88 ^{bd}

Legend: CTRL: control, non inoculated; Starter added (BOX-57: *C. divergens*, *C. maltaromaticum* and *L. sakei*; LAK-23: *L. sakei*; FP-50: *C. divergens* and *C. maltaromaticum*; F-106; *Lacticaseibacillus casei*). Data are the mean of three replicates and are expressed as ratio between peak area of each molecule and peak area of the internal standard (4-methyl-2-pentanol). For each molecule, significant differences according to ANOVA between samples collected at each storage time are indicated by the presence of different letters.



Volatile compound	Factor 1	Factor 2
Pentanal	0.0385	0.0821
Hexanal	0.0188	0.0578
Nonanal	0.0193	0.0180
Benzaldehyde	0.0215	0.0226
Acetone	0.0557	0.0391
2-butanone	0.0038	0.0512
2,3-butanedione	0.0259	0.0165
Methyl isobutyl ketone	0.0805	0.0110
3-penten-2-one, 4-methyl	0.0266	0.0021
Acetoin	0.0001	0.0186
Isopropyl alcohol	0.0397	0.0316
Ethyl alcohol	0.0105	0.0496
1-propanol	0.0158	0.0115
1-penten-3-ol	0.0281	0.0918
1-butanol, 3-methyl	0.0100	0.0689
1-pentanol	0.0080	0.0551
3-buten-1-ol,3-methyl	0.0699	0.0071
cis-2-penten-1-ol	0.0087	0.0807
2-buten-1-ol, 3-methyl	0.0418	0.0188
Hexanol	0.0748	0.0027
1-octen-3-ol	0.0062	0.0690
Heptanol	0.0769	0.0246
Acetic acid	0.0555	0.0336
Propanoic acid	0.0277	0.0474
Butanoic acid	0.0852	0.0048
Butanoic acid, 3-methyl	0.0072	0.0739
Hexanoic acid	0.0804	0.0049
Ethyl acetate	0.0629	0.0061

Figure 3.2 PCA case coordinates for the first two factors explaining the influence of the different bioprotective cultures on the volatile profiles of fish burgers during storage (0, 6, 12 and 18 days). The factor contribution of the aroma compounds to Factor 1 and Factor 2 are reported as table.

3.3.3 Sensory characteristics

Table 3.3 provides the results of a sensory evaluation performed at day 12 of storage. Inoculated burgers were also evaluated on days 18, 24, and 30. Instead, control samples were evaluated only on day 12, as the packages subsequently swelled. Sensory analysis results presented slight changes with the progress of storage. However, on day 30, at the opening of packages, there was a strong ammonia odor, except for the burgers inoculated with LAK-23, which had the best sensory analysis scores during the overall storage period. The inoculation of LAB starter cultures improved the sensory attributes of fish burgers. Indeed, the bioprotective cultures reduced the spoiler activities, such as TVB-N production; consequently, the sensory attributes of sea bass and sea bream burgers were acceptable for up to 12 days of storage (10 days at 4 °C and 20 days at 8 °C). Finally, inoculated burgers did not present odors, flavours, or the sticky white slime indicative of deterioration. In contrast, a sticky, white slime was observed in some uninoculated burgers.

Table 3.3 The sensory panel scores of cooked fish burgers.

Sensory attributes	Day 12				
	CTRL	**BOX-57**	**F-106**	**FP-50**	**LAK-23**
Fermentation	8/12	6/12	4/12	5/12	3/12
Rancid	7/12	4/12	3/12	3/12	2/12
Sweet	2/12	1/12	1/12	1/12	1/12
Pungent	7/12	2/12	3/12	3/12	2/12
Fish	12/12	12/12	12/12	12/12	12/12
Sour	4/12	5/12	5/12	4/12	3/12
Bitter	6/12	4/12	2/12	2/12	2/12
Ammonia	4/12	3/12	1/12	2/12	1/12
Slimes	2/12	0/12	0/12	1/12	0/12
Final scores[a]	5	4	2	3	1

Legend: CTRL: control, non inoculated; Starter added (BOX-57: *C. divergens*, *C. maltaromaticum* and *L. sakei*; LAK-23: *L. sakei*; FP-50: *C. divergens* and *C. maltaromaticum*; F-106; *Lacticaseibacillus casei*). Data: sum of the assessors identi-fiers of the presence of the descriptor out of the total of the assessors. [a]Final scores: the assessors requested to ranked the products within the scale from 1 (excellent) to 4 (worst).

3.4 Discussion

3.4.1 Microbial and physico-chemical parameters

Seafood are one of the most perishable foods, and their shelf-life is limited by enzymatic and microbial spoilage. In fact, the fish matrix is particularly suitable for the development of different types of microorganisms. In addition, during evisceration and filleting, microorganisms from the raw material, manufacturing environment, and human manipulation may contaminate the flesh (Leroi, 2010). Several studies have been carried out to evaluate the effect of antimicrobials other than synthetic additives on the shelf-life of fish burgers (Corbo et al., 2005, 2009; Rico et al., 2020; Uçak et al., 2011). Essential oils and plant extracts have been found to be effective in extending the shelf-life of the aforementioned products by limiting the growth of undesirable microorganisms (Del Nobile et al., 2009; Lucera et al., 2012). However, in some cases, the use of these types of substances may negatively affect the sensory properties of the products (Parlapani, 2021). In the case of minimally processed fish-based products, biopreservation seems to be an attractive alternative and novel method of preservation because of the restrictions for the application of other antimicrobial treatments, such as heat, and the lower consumer acceptance toward synthetic additives (Gόmez et al., 2015). In this study, the effect of different LAB starter cultures on the quality characteristics and shelf-life of sea bass and sea bream burgers was evaluated. The species used as starter cultures in this study are well adapted to the specific characteristics of the fish matrix since they are part of the natural microbiota of this kind of product (González-Rodriguez et al., 2002; Macé et al., 2012; Comi et al., 2016). The role of LAB in seafood is wide and complex; they may have no particular effect or may be responsible for spoilage, and in certain cases, they may exert a bioprotective effect in relation to undesirable bacteria (Bolívar et al., 2021). The bioprotective potential of LAB strains against pathogens, especially *Listeria monocytogenes*, in different seafood products has been extensively studied. The growth of this pathogen was effectively limited by *Latilactobacillus sakei* in cold-smoked sea bass (Iacumin et al., 2021), sea bream (Aymerich et al., 2019), and cold-smoked salmon (CSS) (Tahiri et al., 2009), *Carnobacterium* spp. in CSS (Yamazaki et al., 2003), *Leuconostoc gelidum* and *Lactococcus piscium* in cooked and peeled shrimp and CSS (Matamoros et al., 2009), just to mention a few. However, the use of LAB for improving the microbiological

quality of seafood is probably more insidious than limiting the development of a pathogen, because spoilage is the result of a complex ecosystem composed of different microorganisms. The combination of starter cultures, as occurred in this study, could be useful to exert a wider antimicrobial spectrum. The antibacterial activity of organic acids produced by LAB and their ability to decrease the pH constitute one of the main mechanisms for biopreservation in food products. Despite the inocula performed, the pH reduction in the fish burgers was similar in all the samples. This could be because, in this study, the LAB populations of the control samples reached similar counts to those of the inoculated burgers. In fact, as expected, the LAB population increased during storage since modified atmosphere packaging (MAP) benefits their development, suggesting their suitability to be used to prolong the shelf-life of fresh fish burgers. It is generally recognized that the absence of O_2 and the presence of CO_2 limit or inhibit the growth of gram-negative bacteria such as *Pseudomonas* and *Shewanella*, considered the most important spoilage bacteria in the majority of seafood (Silbande et al., 2016; López-Caballero et al., 2002). However, *Enterobacteriaceae* can develop under these conditions and may play an important role in seafood spoilage (Iacumin et al., 2022). Therefore, controlling the growth of these bacteria will be very effective in shelf-life extension. In contrast, LAB are tolerant to CO_2 and are therefore often found as the dominant organisms in MAP fishery products (Kostaki et al., 2009). Regarding the different microbial population amounts of the burgers, no strong differences were observed between the control and the inoculated samples. These results are in accordance with those of other studies. Brillet et al. (2005) observed a slight inhibition of *Enterobacteriaceae*, yeasts, and endogenous LAB by the inoculation of *C. divergens* V41 in cold-smoked salmon (CSS) only when the initial natural microbial population was lower than 20 CFU/g. In fact, when it was more than 10^4-10^5 CFU/g, as in this study, no effect on the microbial population and physico-chemical characteristics was observed. The similar counts of *Enterobacteriaceae* detected in the samples could also be related to the similar reduction in pH that occurred in all the samples, as previously mentioned. In fact, one of the main inhibitory mechanisms of *Enterobacteriaceae* by LAB is linked to a decrease in pH (Dinardo et al., 2019). Another reason may be that this group of undesired microorganisms is not sensitive to the antimicrobial substances produced by the starter cultures. It is well known that most LAB bacteriocins are not active against gram-negative bacteria because of their complex outer

membrane system (Masuda et al., 2021). Although the starter cultures did not have any effect on the microbial counts, they could have influenced the bacterial community species composition and diversity. In fact, it is common knowledge that the successful inoculation and implantation of starter cultures in a food product limits the growth of endogenous microorganisms and reduces bacterial diversity (Bao et al., 2018). Most likely, in this study, the starter cultures added to the burgers, which had homofermentative metabolism, prevailed over the indigenous LAB and restricted the species variability, limiting the development of heterofermentative LAB, which caused gas formation under the packaging of the uninoculated burgers. In accordance with these results, Cao et al. (2015) observed an influence of the inoculated starter *Lactiplantibacillus plantarum* 1.19 on the composition of Gram-positive bacteria of fresh tilapia fillets, but there was no effect on the Gram-negative bacteria. Heterofermentative LAB have been associated with bloating spoilage by the production of CO_2 in several food products. Different species of the genus *Leuconostoc* were found to be responsible for bloating spoilage in vacuum-packed cooked meat and herring filets (Chenoll et al., 2007; Lyhs et al., 2004). Members of the genus *Weissella* have been related to bulging of broiler or cooked meat packages (Pothakos et al., 2015). In this study, potatoes, added to the fish batter, may have constituted an additional or main fermentable substrate for LAB since the fish matrix was characterized by a low percentage of carbohydrates (0.2–1.5% depending on the species) (Leroi, 2010). This may have contributed to the significant lowering of pH that occurred in all the samples and the development of CO_2 observed in the control. The initial level of TVB-N in the fish burgers was relatively higher than that in similar products reported in other studies (Uçak et al., 2011; Tokur et al., 2004). Moreover, as stated above, at the end of the storage (30 days), the TVB-N values of all the samples considerably exceeded the threshold limit suggested in the abovementioned study, which was 40 mg N/100 g product, although it is dependent on fish species and product type (e.g., whole fish, filets, slaughter method, etc.). At the end of the storage (30 days), the amount detected in the control was significantly higher than that in the inoculated burgers. TVB-N is known as a product of bacterial spoilage and endogenous enzyme action, and its level is often used as an index to evaluate fish quality (Bekhit et al., 2021). The differences between the control and inoculated burgers could not be related to *Enterobacteriaceae* counts, as reported in other studies (Meng et al., 2022; Iacumin et al., 2017). According to the TVB-N values,

sea bass and sea bream burgers supplemented with starter were acceptable for up to 12 days of storage (10 days at 4 °C and 2 days at 8 °C). Indeed, at this time, in burgers supplemented with starter cultures, the level of TVB-N was less than or equal to 40 mg N/100 g. The medium level of TBARS was 2.1 ± 0.5 nmol malondialdehyde/g at 30 days, independent of the treatments; consequently, these values must be accepted. According to several authors (Man & Ramadas, 1998), food products are not rancid when TBARS values are less than 8 nmol/g of the sample, slightly rancid when TBARS is between 9 and 20 nmol/g, and rancid and unacceptable when TBARS is more than 21 nmol/g.

3.4.2 Volatilome

The presence of bioprotective starter cultures and the length of storage changed the volatile profile of the fish burger when compared with the control. The most important accumulated molecules can be derived from the pyruvate metabolism of LAB. When fermentable sugars are scarce or absent, LAB shift to mixed acid fermentation, through which, starting from pyruvate, they can accumulate additional ATP following two different pathways: the pyruvate formate lyase and the pyruvate oxidase, with the production of acetate and ethanol as end products. In addition, the species used in these trials are facultatively heterofermentative and can ferment pentose sugars present; for example, in nucleotides, producing lactic, and acetic acid (Zotta et al., 2017; Gänzle, 2015). In addition, LAB can also obtain pyruvate from other routes, such as citric acid, and amino acids such as serine (Barbieri et al., 2022; Montanari et al., 2018). The presence of pyruvate is also essential to produce diacetyl and acetoin, which have an important effect on the aroma profile. Other compounds that can be derived from the same precursor are acetone, 2-butanone (and its related alcohol 2-butanol), and even butyric acid (Flores et al., 2014; Carballo, 2012). Many other compounds detected are the result of amino acid bacterial metabolism, such as 3-methyl-1-butanol and 3-methyl butanoic acid (derived from leucine metabolism). Interestingly, 3-methyl-3-buten-1-ol and 3-methyl-2-buten-1-ol accumulated more in the presence of bioprotective cultures. According to the results of PCA data, important differences in the volatilome were induced by the presence of bioprotective cultures. The samples supplemented with BOX-57, FP-50, and LAK-23 presented a profile that described the rapid growth and colonization of fish burgers by LAB, with the production of typical molecules derived from their metabolism. The strain *Lacticaseibacillus casei* (F-106) demonstrated slower kinetics, indicating a possible minor

adaptation to the environmental conditions, particularly temperature (4–8 °C). The control presented a clearly different profile. Even if the most important molecules were the same, the kinetics of accumulation were delayed, and in contrast to other samples, after 18 days, ethanol was present in a higher proportion than acetate. These differences indicate that the LAB populations that colonized the burgers in the absence of bioprotective cultures were characterized by a different activation of metabolic routes during storage at refrigeration temperature.

3.4.3 Sensory aspects

The growth of the starter cultures could have had an inhibitory effect on the spoilage activity of the endogenous microorganisms. It was demonstrated that some LAB have the potential to suppress the metabolic activity of certain bacteria, limiting their spoilage potential (Laursen et al., 2006). This type of interaction could be more important than the inhibition of growth, as found in the study carried out by Vasilopoulos et al. (2010), in which carnobacteria limited the growth of *Brochothrix thermosphacta* without suppressing its metabolites. The panellists preferred the burgers inoculated with the starter cultures LAK-23 (*L. sakei*) and F-106 (*L. casei*). These results demonstrated that the inoculated starter cultures improved the sensory attributes of the fish burgers. Similar results were also reported by Comi et al. (2015), in which the use of bioprotective cultures enhanced the microbial quality and sensory properties of meat hamburgers. To fulfill industrial requirements, bioprotective cultures should not adversely affect the sensory attributes of food products.

3.5 Conclusions

The results of this study indicated that the shelf-life of sea bream and sea bass burgers, as determined by the sensory scores and physico-chemical and microbiological data, was 12 days. The inoculation of different starter cultures did not have a particular effect on the microbial populations and physico-chemical characteristics of the burgers. The volatilome changed in the different treatments, and in particular, important differences were induced by the presence of bioprotective cultures. The samples supplemented with BOX-57, FP-50, and LAK-23 presented a profile that described the rapid growth and colonization of fish burgers

by LAB, with the production of typical molecules derived from their metabolism. The sensory attributes of burgers were affected by the presence of the bioprotective cultures, as the odors, flavours, and sticky, white slime indicative of deterioration were not observed. Additionally, inoculated burgers did not demonstrate bloating spoilage. The bioprotective cultures evaluated in this study can potentially extend the shelf-life and improve the sensory properties of fish burgers, contributing to the reduction of food waste in the fish supply chain.

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Part 2

Lactic Acid Bacteria
for
Human Health Applications

4. Cell-surface properties of lactic acid bacteria for health applications

4.1 Introduction

The knowledge of the properties of lactic acid bacteria (LAB) cell wall is fundamental for the development of cultures for technological and health applications since the interaction of the bacterial cell with the environment, the other microorganisms and the host depends on them. Bacterial cell is surrounded by a phospholipidic double layer, which is itself enclosed by the cell wall that is the frontier between the bacteria and the external environment. It follows from this that cell surface properties depend on the composition, structure, and component organization of the cell wall. As it is well known, bacteria are primarily classified on the basis of their cell wall composition and organization in two main groups: Gram-positive and Gram-negative bacteria. Gram-positive bacteria and therefore LAB cell wall is composed mainly of peptidoglycan decorated with teichoic acids, polysaccharides, and proteins (Moreillon & Majcherczyk, 2003). The main physicochemical properties of cell-surface usually investigated are: i) autoaggregation, aggregation of bacteria belonging to the same strain; ii) coaggregation, aggregation of bacteria belonging to different strains; iii) hydrophobicity; tendency to adhere to apolar molecules; iv) adhesion, attachment to different surfaces. Several studies have included the assessment of these properties in the preliminary screening for the selection of potentially probiotic bacteria (Krausova et al., 2019; Tuo et al., 2013; Tomičić et al., 2022). Bacterial adhesion and colonization in a wide variety of environments are considered survival strategies that provide a competitive advantage (Hunt et al., 2004). Consequently, the intestinal epithelium adhesion ability is a main criterion for the selection of probiotics and it is included in the "Guidelines for Evaluation of Probiotics in Food" published by the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) (Araya et al., 2002). Along with adhesion and the other strictly related properties, stress tolerance and antimicrobial activity are evaluated and, when necessary, a safety assessment is also carried out. Some additional requirements may be the host-associated functional properties, which include anticancer, anti-cholesterol, anti-obesity,

anti-diabetic, and immunomodulatory activities (de Melo Pereira et al., 2018). The reason why adhesion ability is included in the different steps necessary for the selection of probiotic strain is that high adhesion ability would increase the possibility of intestinal colonization. However, it has been demonstrated that probiotics generally do not colonize the intestine permanently (Alander et al., 1997; Fuller & Gibson, 1997). In any case, high adhesion ability may lead to an increase in the residence time of culture in the gastrointestinal tract (GIT), resulting in transient colonization that would increase the possibility of exerting a beneficial effect on the host, for instance, immunomodulatory activity and other proximity-dependent positive effects are favoured by high adhesion ability (Suez et al., 2019). Moreover, high adhesion ability could be a possible mechanism for the exclusion of pathogens through the competition of binding sites (Collado et al., 2005). The chemical properties of bacterial cell surface constituents and their structural organization within the cell wall, as well as the conformation of the surface macromolecules, contribute to varying degrees of adhesion ability. In the literature, the studies based on the quantification of the adhesion capacity of LAB are present in considerable numbers, meanwhile those concerning the mechanisms involved in this ability are less spread. However, it is common knowledge that bacteria use different structures for the adhesion to intestinal epithelial cells including flagella, fimbriae, pili, mucus binding, fibronectin-binding, and surface-layer proteins, and teichoic acid (Alp & Kuleaşan, 2019). Capsular exopolysaccharides, meaning exopolysaccharides that remain attached to the peptidoglycan of the cell wall, also contribute to the adhesion capacity of bacteria able to synthesize (Nachtigall et al., 2020). However, all these elements may have a different impact on adhesion. It was noticed that the removal of S-layer proteins had more effect on the aggregation ability rather than on adhesion ability, suggesting that those proteins had a primary role in aggregation whereas the adhesion ability may depend to a different extent also by other cell wall structures (Alp et al., 2020). Besides the influence of the different elements present in the cell wall, adhesion ability may depend on other extrinsic factors including intestinal mucus and environmental conditions. Adhesion to enterocytic cells can be specific when occurs at specific receptor sites and non-specific when the overall cell surface components are involved (Ljungh & Wadstrom, 2006). In the latter case, it has been demonstrated that cell wall hydrophobicity plays a major role. This characteristic is shown to be associated also to other interesting properties of LAB such as their butanol

tolerance and acrylamide absorption (Petrova et al., 2019; Shen et al., 2019). Moreover, this characteristic contributes to the bacterial detoxification potential of some mycotoxins, for instance, high cell wall hydrophobicity facilitates the detoxification of ochratoxin A and aflatoxin B1 via a hydrophobic bond (Abedi et al., 2022; Nasrollahzadeh et al., 2022). As for aggregation and adhesion, hydrophobicity of bacterial cells depends on the characteristics of the cell surface. The most relevant macromolecules that cover the peptidoglycan layer of LAB cell wall are lipo- and teichoic acids, polysaccharides, and proteins (Delcour et al., 1999). On the one hand, components like proteins and (lipo-) teichoic acids confer hydrophobic properties on LAB surface. On the other hand, polysaccharides render the LAB surface hydrophilic (Schär-Zammaretti & Ubbink, 2003). Modification of bacterial surface, and subsequently the modification of its properties, can occur by changes in temperature, growth phase, and growth medium composition (Deepika et al., 2009; Zeraik & Nitschke, 2012). Along with adhesion, coaggregation ability of LAB could contribute to the exclusion of enteropathogenic bacteria in the GIT. Coaggregation between probiotics and pathogens has been investigated in different studies using *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes*, *Cronobacter sakazakii*, *Campylobacter jejuni*, and *Bacillus cereus* as main target pathogens (Tatsaporn & Kornkanok, 2020; Gómez et al., 2016; Campana et al., 2017). This ability may be relevant also in other fields of application as, for instance, coaggregation ability can inhibit pathogenic biofilm formation (Merino et al., 2019). Among LAB, the strains belonging to the *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei*, and *Lacticaseibacillus rhamnosus* species which have been involved in food fermentation (e.g., vegetables and dairy products) and they are frequently used as probiotics, for these reasons, they are among the most studied LAB species. Many of the studies regarding these species have been focused on the characterization of the probiotic properties of newly isolated strains with the aim to select potential probiotics other than those currently available on the market. Several studies in which a higher inter-strain variability of the probiotic properties have been detected (Muñoz-Provencio et al., 2009). However, some authors stated that some cell surface properties of lactobacilli may be, at least in part and under certain experimental conditions, specific to the bacterial species and the differences among the species are likely to be due to some particular differences in cell wall chemical composition (Pelletier et al., 1997). The aim

of this study was to assess the cell surface properties (adhesion, autoaggregation, hydrophobicity, and coaggregation) of 74 LAB strains.

4.2 Materials and methods

4.2.1 Bacterial strains and growth conditions

A total of 74 strains (Table 4.1) were assessed for their cell surface properties. The strains were stored at -80 °C as 30% (vol/vol) glycerol stock-cultures in de Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Milan, Italy) and routinely propagated (1% w/v) in MRS broth for 24 h at 37 °C before the analysis.

Table 4.1 *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei* and *Lacticaseibacillus rhamnosus* strains used in this study and their relative isolation sources.

Origin	**Identification**
Raw milk	*L. paracasei*: P1E6[3], P2P3[3]
Cheese	*L. casei*: DSM20011[1] *L. paracasei*: LMG25880[1], DBPZ0421[8], DBPZ0422[8] DBPZ0434[8], DBPZ0435[8], DBPZ0476[8], DBPZ0477[8,] DBPZ0478[8], M348[8], DSG03[8], TH1229[9], TH406[9], TMW1.1444[6], R61[13], F17[13], DBPZ0450[8] *L. rhamnosus*: PRA204[5], PRA232[5], PRA331[5], DBPZ0428[8], DBPZ0445[8], DBPZ0448[8], CI4362[12], CF377[12], D44[13] L9[9], L47[9], DBPZ0446[8]
Fermented sausages	*L. rhamnosus*: CTC1676[14], 2220[15]
Sourdough	*L. paracasei*: DBPZ0572[8], Q2[8] *L. casei*: DBPZ0571[8]
Beer	*L. paracasei:* TMW 1.300[6]
Wine, must, and cellar equipment's	*L. paracasei*: LMG11961[1], LMG11963[1], LMG13731[1], B161[11], B350[11], LMG13717[1]
Human	*L. casei*: LMG23516[1], N811[16] *L. paracasei*: DSM20020[2], LMG11459[1], LMG23518[1], LMG23523[1], LMG24132[1], DSM4905[2], LMG23538[1], LMG24098[1], N76[16], DBTA34[18], LMG24101[1] *L. rhamnosus*: DBTC4[10], N94[16], N95[16], N83[16], N812[16], N1110[16], TMW1.1538[6], N201[16], N209[16], N715[16], DBTA86[18], N2010[16], N173[16], N132[16], N131[16]
Unknown	*L. rhamnosus*: DSM20021[2], DBPZ0524[8], HA111[4]

[1]LMG: BCCM/LMG, Belgian Co-ordinated Collections of Micro-organisms (BCCM™), Belgium.
[2]DSM: DSM, Deutsche Sämmlung von Mikroorganismen und Zellkülturen, Braunschweig, Germany
[3]Dipartimento di Agraria, Università degli Studi di Sassari, Sassari, Italy.
[4]Harmonium International Inc., Mirabel, Canada.
[5]Dipartimento di Scienze Agrarie e degli Alimenti, Università delgi Studi di Modena e Reggio Emilia, Italy
[6]Lehrstuhl für Technische Mikrobiologie, Technische Universität München, Freising, Germany

[7] Dipartimento di Biotecnologie, Università degli Studi di Verona, Verona, Italy
[8]Scuola di Scienze Agrarie, Alimentari e Ambientali, Università degli Studi della Basilicata, Potenza, Italy
[9]Veneto Agricoltura, Istituto per la Qualità e le Tecnologie Agroalimentari, Thiene (VI), Italy
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[13]Dipartimento di Scienze delle Produzioni Agrarie e Agroalimentari, Università degli Studi di Catania, Catania, Italy.
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[15]Dipartimento di Scienze degli Alimenti, Università degli studi di Udine, Udine, Italy.
[16]Dipartimento di Agricoltura, Ambiente e Alimenti, Università degli Studi del Molise, Campobasso, Italy.

E. faecalis ATCC 29212 was used for testing the coaggregation ability of LAB strains with pathogens. The strain was stored at -80 °C as 30% (vol/vol) glycerol stock-culture in Brain Heart Infusion (BHI) broth (Oxoid, Milan, Italy) and routinely propagated in BHI broth for 24 h at 37 °C before the analysis.

4.2.2 SW480 cell culture

The human colorectal adenocarcinoma cell line SW480 was kindly provided by the Department of Medicine of the University of Udine (Italy). The cell line was cultured in Dulbecco's Modified Eagles Medium (Stable cell DMEM; Sigma-Aldrich, Milan, Italy) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Milan, Italy) and maintained in 25 cm^2 or 75 cm^2 culture flasks (Sarstedt, Nümbrecht, Germany) in a humidified-atmosphere with 5% CO_2 and 95 % air in an incubator (Euroclone, Milan, Italy) at 37 °C. Every two days confluent cultures were split 1:3, after washing with phosphate buffered saline (PBS; pH 7.4, Sigma-Aldrich, Milan, Italy) using trypsine-EDTA (SAFC Biosciences, Lenexa, KS, USA).

4.2.3 Adhesion of bacteria to SW480 cell line

The adhesive capability of the strains was assessed by using SW480 cells as an intestinal epithelial cell model. SW480 cells were seeded in a 24 well tissue culture plates at a concentration of $3.0x10^5$ cell/well and incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C until confluence. All the tested strains were cultured in MRS broth for 24 h at 37 °C. After the incubation, the lactic acid bacteria cultures were centrifuged at 9,700 x *g* for 10 min and the pellets were washed twice with PBS buffer. Bacterial suspensions were resuspended in PBS buffer and the absorbance at a wavelength of 600 nm was adjusted to 0.1, that correspond approximately to 10^7 cell/mL (previously determined; data not shown). One

milliliter of each suspension was centrifuged (9,700 x *g*; 10 min) and resuspended in the same volume of fetal bovine serum and antibiotic free DMEM. The exact number of bacterial cells contained in the suspension was determined by the serial dilution method in de Man, Rogosa, and Sharpe agar medium (MRS, Oxoid, Milan, Italy). Prior to the assay, the SW480 monolayers were washed twice with PBS and thereafter, 1 mL of the bacterial suspension was added to each well. Plates were incubated for 3 h at 37 °C with 5% CO_2 in humidified atmosphere. After incubation, supernatants were discarded, and each well was washed twice with PBS to remove the non-attached bacteria. To release attached bacterial cells, the SW480 monolayer was treated with 1 mL of 1% Triton X-100 (Sigma-Aldrich, Milan, Italy) for 20 min. The bacteria recovered from each well were centrifuged (9,700 x *g*; 10 min), washed with 1 mL of PBS buffer, and resuspended in the same volume of saline-peptone water (0.9% NaCl; 0.1% bacteriological peptone, Oxoid, Milan, Italy). Serial dilutions of the suspensions were plated onto MRS plates and incubated at 37 °C for 48 h to quantify adhering bacteria. Adhesion was expressed as the percentage of number of adhered bacteria to the total bacteria used for the experiment and calculated according to the following equation:

$$Adhesion\ (\%) = \left(\frac{B_1}{B_0}\right) * 100$$

where B_0 and B_1 are the initial and final count of bacteria expressed as CFU/mL, respectively. The adhesion assay was conducted in triplicate for each strain.

4.2.4 Autoaggregation

The ability of LAB to autoaggregate was evaluated according to the methodology described by Pammi et al. (2021). LAB strains were grown at 37 °C for 24 h in MRS broth. The bacteria cells were harvested by centrifugation at 9,700 x *g* for 10 min, washed twice with phosphate-buffered saline PBS (pH 7.4), and resuspended in the same buffer. Absorbance at an optical density of wavelength of 600 nm (A_{600}) was adjusted to 0.10 ± 0.01 to standardize the number of bacteria (10^7 CFU/mL). The suspension was then incubated at 37 °C for 24 h and the absorbance was measured before and after the incubation. Autoaggregation percentage was determined using the following equation:

$$Autoaggregation\ (\%) = \left(1 - \frac{A_t}{A_0}\right) \times 100$$

where A_0 represents absorbance at t = 0 and A_t the absorbance at t = 24h.

4.2.5 Cell surface hydrophobicity

Cell surface hydrophobicity was determined by measuring bacterial adhesion to hydrocarbons (BATH) using xylene as a solvent. The assay was performed according to the procedure described by Doyle and Rosenberg (1995) with some modifications. Bacterial suspension was prepared as described for auto aggregation assay. Three milliliters of cell suspension were then added to one milliliter of xylene. The two-phase system was thoroughly mixed by vortex for 1 min and incubated for 3 h at 37 °C to allow the separation of the two phases. The lower aqueous phase was carefully removed and the A_{600} was measured (A_t).

The hydrophobicity was calculated according to the following equation:

$$Hydrophobicity\ (\%) = \left(1 - \frac{A_t}{A_0}\right) \times 100$$

where A_0 represents absorbance at 0 h and A_t the absorbance after the indicated incubation time.

4.2.6 Coaggregation

Coaggregation ability of LAB with *E. faecalis* ATCC 29212 was assayed as reported by Tuo et al. (2013). Equal volumes of cell suspensions of pathogen and probiotic, prepared as for the autoaggregation assay (*4.2.4*), were mixed by vortex for 10 s. The mixture was incubated at 37 °C for 24 h. Coaggregation percentage was calculated as follows:

$$Coaggregation\ (\%) = \frac{\frac{A_P + A_{LAB}}{2} - A_{mix}}{\frac{A_p + A_{LAB}}{2}} \times 100$$

Where A_p and A_{LAB} represented the absorbance of the pathogen and the LAB strain, respectively, and A_{mix} represented the absorbance of the mixed culture after the indicated incubation time.

4.2.6 Unsupervised clustering algorithms

The heat-map and clustering analysis were performed using pheatmap (version 1.0.12) and ggfortify (version 0.4.14) R packages (R Core Team, 2021) with Euclidean distance and Ward method.

4.2.7 Statistical analysis

The statistical analyses were performed using R software version 4.1.2 (R Core Team, 2021). The normality of data distribution was assessed with Shapiro-Wilk test. Kruskal–Wallis H test was used to assess the differences between strains. Dunn test was considered for post hoc testing with Benjamini Hochberg corrections for multiple comparisons. Spearman's correlation coefficient was used for the determination of correlation between variables.

4.3 Results and discussion

In this study, the cell surface properties of 4 *L. casei*, 38 *L. paracasei*, and 32 *L. rhamnosus* strains, isolated from different food and human sources, were assessed. Lactic acid bacteria belonging to these species are commonly used in the food industry as well as successfully used as probiotics. As aforementioned, adhesion ability is regarded as an important property to be evaluate during the probiotic selection procedure. Studying bacterial adhesion *in vivo* is difficult and therefore *in vitro* assessment is usually performed. In particular, adhesion ability was evaluated using intestinal cell lines, mucin-producing cell lines, as well as immobilized intestinal mucus (Coconnier et al., 1992; Nishiyama et al., 2016). In fact, bacterial cell attachment in the intestine occurs to epithelial cells but also to their protective layer of mucus, which is considered an important site for bacterial adhesion and colonization (Kirjavainen et al., 1998). Unfortunately, the *in vitro* adhesion assay does not take into account several factors that would influence the LAB adhesion such as the host defense system, resident microbiota, and GIT peristaltic contractions (Servin, 2004). SW480 cell line is not so frequently used for adhesion assay compared to other human colon adenocarcinoma cell lines (Caco-2 and HT-29), although some studies are present in the literature (Wang et al., 2018; Zárate et al., 2017).

4.3.1 Adhesion of bacteria to SW480 cell line

In this study, as shown in Table 4.2, adhesion capacity resulted to be highly variable depending on the strain tested and this agrees with other studies (Garcia-Gonzalez et al., 2018; Campana et al., 2017; Tuo et al., 2013). *L. paracasei* LMG 24098 was not able to adhere to the cell line whereas *L. paracasei* LMG24101 resulted to be the strain with the highest adhesion ability (41.8%). Along with the latter strain, also *L. rhamnosus* DSM 20021 (40%),

L. rhamnosus L9 (37.8%), *L. paracasei* R61 (35.9%), *L. casei* DSM 20011 (33%) presented a high adhesion ability compared to the others. Within the range of 0-41.8%, only the 10% of the strains had an adhesion ability higher than 20% and approximately the 60% of the strains had an adhesion ability lower than 10%. Adhesion ability lower than 10% for strains belonging to the same species tested in this study was detected frequently also by other authors (Monteagudo-Mera et al., 2012; Bertazzoni-Minelli et al., 2004; Tuomola & Salminen, 1998). Tuo et al. (2013) found an adhesion ability of *Lactiplantibacillus plantarum* strains between 24 and 53% whereas that of *L. casei* and *L. rhamnosus* strains were lower than 10%. It must be taken into account that the studies mentioned in this section and used for the comparison used different colorectal adenocarcinoma cell lines. Comparing the percentages of adhesion ability obtained in this study with those of other studies appears to be not so useful since it has been demonstrated that bacteria adhere differently depending on the cellular model used (Coconnier et al., 1992). The components of bacterial cell wall may vary considerably depending on the species and even on the strain. Some authors demonstrated that *L. paracasei* BGSJ2-8 was able to produce a strain-specific exopolysaccharide responsible for the great part of the adhesion ability to epithelial intestinal cells (Živković et al., 2016). Collado et al. (2007) observed that the combination of different probiotic strains may have synergistic adhesion effects besides the possibility of having additional beneficial effects. Several studies present in the literature assessed the adhesion ability of lactobacilli belonging to the species tested in this study. The adhesion ability seems to be not correlated with the isolation sources (food or human), these results are confirmed also by Muñoz-Provencio et al. (2009).

Table 4.2 Mean value ± SD of cell surface properties of LAB strains.

	Autoaggregation (%)		Hydrophobicity (%)		Coaggregation (%)		Adhesion (%)	
LMG11459	54.5^{defghijkl}	1.47	75.6^{hijklmno}	11.14	-2.1 ^{ghijklm}	2.30	14.1 ^{cdefgh}	3.79
LMG11961	31.5^{stuvwx}	2.54	11.7^{xyz}	1.27	0.8 ^{bcdefghijklm}	0.76	12.8 ^{defgh}	5.00
LMG11963	27.8^{uvwxy}	4.45	6.3^{yz}	2.45	1.2^{bcdefghijklm}	1.09	13.6 ^{cdefgh}	4.29
LMG13717	32.2^{stuvwx}	2.30	96.2^{abcd}	1.76	1.1 ^{bcdefghijklm}	0.44	19.3 ^{abcdefgh}	17.86
LMG13731	35.9^{qrstuvw}	14.78	96.6^{abc}	0.73	-0.5 ^{efghijklm}	2.80	6.5^{fgh}	0.59
LMG23518	27.5^{uvwxy}	3.59	97.6^{abc}	2.04	1.0^{bcdefghijklm}	0.91	16.7 ^{bcdefgh}	1.67
LMG23523	68.5^{bc}	3.47	10.4^{xyz}	1.66	3.7^{abcdef}	2.77^{efgh}	10.9^{efgh}	3.62
LMG23538	40.1^{nopqrstu}	1.48	93.9^{abcde}	0.56	0.3 ^{cdefghijklm}	0.50	1.7^{h}	0.37
LMG24098	51.0^{fghijklmno}	0.00	78.1^{fghijklm}	1.04	0.6 ^{cdefghijklm}	3.88	0.0^{h}	0.01
LMG24101	69.4^{b}	0.93	65.7^{mnopqr}	12.86	2.5^{bcdefghij}	0.75	41.8^{a}	26.29
LMG24132	69.1^{b}	2.15	83.9^{bcdefghijk}	0.77	1.9^{bcdefghijkl}	0.80	2.7^{h}	1.43
LMG25880	64.4^{bcde}	0.99	71.9^{jklmnopq}	0.57	6.4^{ab}	1.29	2.0^{h}	1.36
LMG23516	64.5^{bcde}	3.60	81.3^{defghijkl}	5.45	2.7^{bcdefghi}	2.61	6.4^{fgh}	1.54
DSM 4905	59.1^{bcdefghi}	5.63	90.1^{abcdefgh}	2.96	6.0^{abc}	2.64	3.6^{gh}	1.31
DBPZ0421	65.2^{bcd}	4.48	97.8^{ab}	1.08	-2.5 ^{hijklm}	2.08	7.9^{fgh}	4.73
DBPZ0422	65.0^{bcd}	3.18	95.7^{abcd}	0.57	-3.3^{klm}	1.28	22.6^{abcdefgh}	11.54
DBPZ0428	15.3^{y}	2.70	98.0^{ab}	0.75	8.6^{a}	0.42	3.7^{gh}	1.74
DBPZ0434	67.7^{bc}	2.49	47.5st	6.93	-1.4 ^{efghijklm}	0.85	1.7^{h}	0.96
DBPZ0435	39.2^{nopqrstuv}	8.78	25.0^{vwx}	3.31	-0.1 ^{defghijklm}	1.91	21.0^{abcdefgh}	13.34
DBPZ0445	57.4^{bcdefghij}	3.89	88.5^{abcdefgh}	0.96	0.9 ^{bcdefghijklm}	0.39	1.8^{h}	1.32
DBPZ0446	61.2^{bcdefgh}	0.56	92.9^{abcdef}	1.47	-0.7 ^{efghijklm}	0.44	28.9^{abcdef}	2.48
DBPZ0448	15.7^{y}	4.04	97.6^{abc}	1.12	5.3^{abcd}	1.89	9.1^{efgh}	3.97
DBPZ0450	38.7^{opqrstuv}	1.45	92.7^{abcdef}	1.45	-0.9 ^{efghijklm}	0.78	12.8 ^{defgh}	0.94
DBPZ0476	66.3^{bcd}	0.69	94.0^{abcde}	3.15	-2.8 ^{ghijklm}	2.53 ^{efgh}	10.8	2.67
DBPZ0477	63.8^{bcdef}	2.37	94.0^{abcde}	1.96	0.8 ^{bcdefghijklm}	1.84	11.3 ^{defgh}	0.63
DBPZ0478	67.7^{bc}	0.57	94.7^{abcde}	3.02	2.9^{bcdefgh}	0.40	3.8^{gh}	1.45
DBPZ0524	62.2^{bcdefg}	2.04	94.9^{abcde}	1.02	2.9^{bcdefg}	2.27	2.5^{h}	0.72
DBPZ0571	21.0^{xy}	3.49	57.1^{qrs}	7.88	4.2^{abcde}	1.66	6.3^{fgh}	1.95
DBPZ0572	28.0^{uvwxy}	1.63	16.3^{wxy}	3.25	-3.5^{lm}	4.64	8.2^{efgh}	0.51
DBTA86	62.4^{bcdefg}	1.18	51.0^{rst}	9.87	1.3^{bcdefghijklm}	0.20	5.1^{fgh}	1.94
DBTC4	66.0^{bcd}	1.56	58.2^{qrs}	8.35	-3.1^{jklm}	0.81	3.3^{gh}	1.36
DBTA34	61.6^{bcdefgh}	1.88	59.2^{pqrs}	3.75	-0.1 ^{defghijklm}	1.51	4.1^{fgh}	2.19
Q2	59.4^{bcdefghi}	1.98	75.6^{hijklmno}	7.30	0.2 ^{defghijklm}	0.70	5.6^{fgh}	3.18
DSG03	56.2^{cdefghijk}	0.95	62.9^{nopqr}	8.14	0.0 ^{defghijklm}	0.82	3.6^{gh}	1.11
M348	41.8^{lmnopqrst}	0.00	1.2^{yz}	0.26	0.9 ^{bcdefghijklm}	1.36	4.3^{fgh}	0.82
PRA204	44.2^{klmnopqrs}	2.49	5.0^{yz}	0.99	0.6 ^{cdefghijklm}	1.48	9.6^{efgh}	5.77
PRA232	41.0^{nopqrst}	2.41	8.0^{yz}	0.60	0.6 ^{cdefghijklm}	0.45	10.3^{efgh}	7.94
PRA331	42.2^{lmnopqrst}	1.23	6.2^{yz}	1.70	0.0 ^{defghijklm}	0.79	11.5 ^{defgh}	3.90
CI4362	38.1^{pqrstuvw}	13.73	98.4^{ab}	0.69	3.8^{acdef}	2.16	5.2^{fgh}	2.65
CF377	38.8^{opqrstuv}	2.38	7.9^{yz}	3.31	0.8 ^{bcdefghijklm}	0.73	9.0^{efgh}	5.40
R61	39.2^{nopqrstuv}	3.66	2.4^{yz}	0.60	0.5 ^{cdefghijklm}	1.59	35.9^{abcd}	19.78
F17	39.3^{nopqrstuv}	2.29	8.6^{yz}	3.02	0.6 ^{cdefghijklm}	1.86	16.3 ^{bcdefgh}	2.50
D44	37.9^{pqrstuvw}	0.72	98.3^{ab}	0.72	-2.9^{ijklm}	4.99	4.7^{fgh}	2.60
TH406	43.5^{klmnopqrst}	0.00	5.1^{yz}	1.66	0.4 ^{cdefghijklm}	1.14	19.7 ^{abcdefgh}	6.39
TH1229	41.9^{lmnopqrst}	3.88	4.7^{yz}	1.24	1.2^{bcdefghijklm}	1.37	5.6^{fgh}	1.34

L9	43.6^{klmnopqrst}	2.77	5.6^{yz}	1.12	0.5 ^{cdefghijklm}	0.53	37.8^{abc}	11.11
L47	83.0^{a}	2.31	98.9^{a}	0.00	-2.2 ^{ghijklm}	1.18	9.4^{efgh}	3.65
N76	52.0^{efghijklmn}	0.98	73.2^{ijklmnop}	1.50	0.3 ^{cdefghijklm}	0.69	5.0^{fgh}	1.55
N715	54.1^{defghijkl}	2.75	80.1^{efghijklm}	4.34	-0.5 ^{efghijklm}	1.29	0.5^{h}	0.04
N94	60.7^{bcdefgh}	1.51	92.4^{abcdefg}	3.75	-0.9 ^{efghijklm}	1.51	5.7^{fgh}	2.95
N95	60.4^{bcdefgh}	0.61	95.4^{abcd}	2.19	1.1 ^{bcdefghijklm}	1.71	3.1^{gh}	2.06
N83	68.7^{bc}	1.01	94.3^{abcde}	1.54	-4.1^{m}	0.74	1.7^{h}	1.38
N811	63.5^{bcdef}	1.92	93.3^{abcde}	1.92	-1.6 ^{fghijklm}	0.94	3.2^{gh}	0.34
N201	41.1^{mnopqrst}	2.80	86.9^{abcdefghij}	0.93	1.5^{bcdefghijklm}	1.28	0.9^{h}	0.59
N209	49.7^{ghijklmnop}	0.53	82.7^{cdefghijk}	2.33	2.3^{bcdefghijk}	1.13	4.7^{fgh}	0.32
N132	49.5^{ghijklmnop}	4.95	4.8^{yz}	1.02	0.1 ^{defghijklm}	1.10	5.5^{fgh}	0.35
N812	57.1^{bcdefghij}	0.95	95.6^{abcd}	3.34	0.7 ^{bcdefghijklm}	2.10	3.7^{gh}	0.45
N173	49.2^{hijklmnop}	3.22	40.7^{tu}	1.40	-0.9 ^{efghijklm}	0.35	12.7 ^{defgh}	3.00
N1110	45.7^{jklmnopqr}	3.69	70.6^{klmnopq}	3.25	1.0 ^{bcdefghijklm}	1.08	4.2^{fgh}	1.10
N131	42.9^{lmnopqrst}	0.00	7.8^{yz}	5.62	0.6 ^{cdefghijklm}	0.22	15.1 ^{bcdefgh}	2.15
P1E6	49.7^{ghijklmnop}	4.82	4.1^{yz}	3.06	2.2^{bcdefghijk}	1.99	19.0 ^{abcdefgh}	14.76
P2P3	47.1^{ijklmnopq}	1.06	9.5^{yz}	2.31	1.1 ^{bcdefghijklm}	0.43	7.2^{fgh}	4.51
N2010	54.0^{defghijklm}	2.77	38.5^{tuv}	3.98	0.3 ^{cdefghijklm}	0.38	3.6^{gh}	0.07
B161	38.6^{opqrstuv}	3.04	77.6^{ghijklmn}	7.89	-0.2 ^{defghijklm}	1.94	19.1 ^{abcdefgh}	10.94
B350	49.0^{hijklmnop}	2.91	62.2^{opqrs}	0.70	-0.6 ^{efghijklm}	0.57	4.2^{fgh}	0.42
CTC1676	32.9^{rstuvwx}	0.69	95.6^{abcd}	1.37	-0.3 ^{defghijklm}	0.71	12.5^{defgh}	2.21
HA111	33.7^{rstuvwx}	3.78	39.2^{tuv}	11.12	-0.4 ^{defghijklm}	0.36	9.0^{efgh}	4.42
2220	31.3^{tuvwx}	5.80	26.7^{uvw}	6.94	-0.6 ^{efghijklm}	1.25	18.3 ^{abcdefgh}	2.03
TMW 1.1444	35.5^{qrstuvw}	1.63	94.0^{abcde}	5.86	0.7 ^{cdefghijklm}	0.63	12.4 ^{defgh}	3.42
TMW 1.1538	41.7^{lmnopqrst}	4.23	91.3^{abcdefg}	1.94	-1.5 ^{fghijklm}	1.87	14.5 ^{cdefgh}	4.50
TMW 1.300	35.8^{qrstuvw}	4.45	87.2^{abcdefghi}	5.57	-1.0 ^{efghijklm}	1.06	27.9^{abcdefg}	15.00
DSM 20011	33.3^{rstuvwx}	3.97	97.4^{abc}	2.25	-0.5 ^{efghijklm}	1.36	33.0^{abcde}	19.67
DSM 20020	25.3^{wxy}	2.11	66.7^{lmnopq}	5.40	0.0 ^{defghijklm}	0.36	11.0 ^{defgh}	9.00
DSM 20021	27.0^{vwxy}	1.93	62.0^{opqrs}	6.70	1.2 ^{bcdefghijklm}	0.71	40.0^{ab}	20.00

$^{a–z}$ Within column, values followed by the same letters are not significantly different ($p<0.05$).

4.3.2 Autoaggregation

L. rhamnosus DBPZ0428 and *L. rhamnosus* L47 were the strains with the lowest (15.3%) and the highest (83.0%) ability to self-aggregate, respectively (Table 4.2). It must be noted that the range of autoaggregation values for the majority of the strains tested is more restricted than that constituted by the extreme values, finding about 90% of the strains located in the range from 30 to 70%. Autoaggregation, along with hydrophobicity, is considered an additional assay to study the adhesion properties of bacteria since most of the studies observed a correlation between these properties. However, this analysis would deserve a most important role in the probiotic selection procedure. It was observed that autoaggregation was higher at lower pH (<4.3) compared to that detected at higher pH (Guglielmetti et al., 2009).

Bacteria aggregates formed at low pH could constitute a survival mechanism that provides resistance against gastric pH. In the intestine, the higher pH could lead the single bacterial cells to be released and subsequently to be available for adhesion. In future studies, bacteria should be subjected to lower pH before being tested for adhesion assay in order to better reproduce *in vivo* conditions and assess if, in this condition, a high autoaggregation corresponds to a high adhesion rate. Changes in the autoaggregation ability may be related to the cell surface charge which was observed to be pH-dependent. Pelletier et al. (1997) observed that the *Lactobacillus* strains studied were in general slightly negatively charged at alkaline pH. The negative correlation found between adhesion and autoaggregation (ρ=-0.315, $p<0.01$; Table 4.3) agrees with other authors who stated that autoaggregation may hide the surface constituents involved in the adhesion to epithelial cells, resulting in a lower adherence probability (Guglielmetti et al., 2009). However, in literature are present some studies, which found a positive correlation between the two properties (Guan et al., 2020; Del Re et al., 2000). No correlation was also found by some authors, suggesting that different mechanisms and different surface molecules are implicated in aggregation and adhesion (Chaffanel et al., 2018). It can be concluded that the complex adhesion process cannot be summarized solely on the basis of some bacterial surface properties.

4.3.3 Cell surface hydrophobicity

BATH is the most used method for measuring the hydrophobicity of the cell surface of LAB being the hydrocarbons hexadecane, xylene, and toluene as the most used apolar solvents (Diana et al., 2015). High variability of the values of adhesion to xylene (hydrophobicity) was observed (Table 4.2), according to other studies, which detected a wide heterogeneity among strains of lactobacilli of the same species (de Souza et al., 2019; Krausova et al., 2019). The highest hydrophobicity was observed for *L. rhamnosus* L47 (98.9%) followed by *L. rhamnosus* CI4362 (98.4%), *L. rhamnosus* D44 (98.3%), and *L. rhamnosus* DBPZ0428 (98.0). *L. paracasei* M348 (1.2%) and *L. paracasei* R61 (2.4%) were the strains with the lowest hydrophobicity. As suggested by other authors, strains with hydrophobicity values higher than 70% were arbitrarily classified as hydrophobic (Martin et al., 1989; Nostro et al., 2004). Accordingly, 40 strains, corresponding to about 50% of the total strains tested, resulted to be hydrophobic. It has also been suggested that high hydrophobicity would correspond to a high adhesion capacity to intestinal cells (Zare¸ ba et al., 1997). However, hydrophobicity

and adhesion to enterocytes demonstrated a negative correlation (ρ=-0.178, $p<0.01$; Table 4.3), according to Krausova et al. (2019) and Schillinger et al. (2005). In other studies, hydrophobicity did not correlate with adhesion capacity and probably this property is not a prerequisite for adhesion (Zago et al., 2011; García-Cayuela et al., 2014; Wang et al., 2018). As previously mentioned, hydrophobic interaction plays a major role in the non-specific adhesion to enterocytes, however, other mechanisms are involved and the non-specific adhesion constitutes only the first interaction between the bacteria and the host (Ljungh & Wadstrom, 2006). No correlation was observed between hydrophobicity and autoaggregation (ρ=0.126, p=0.06; Table 4.3), however, it is worth noting that the strain *L. rhamnosus* L47 with the highest value of autoaggregation had also the highest values of hydrophobicity. This positive relationship has been noted in other studies (Collado et al., 2008; Taheri et al., 2009).

4.3.4 Coaggregation

In this study, coaggregation between lactobacilli and *E. faecalis* was also investigated (Table 4.2). Although some species that constitute intestinal microflora, in many cases may have a defined role (beneficial or deleterious), *E. faecalis* has a controversial role. It has been noticed that the population of the species *E. faecalis* in the fecal microflora of people with colorectal cancer (CRC) is higher compared to healthy people (Wang et al., 2015). *Enterococcus faecalis* leads to the development of chromosomal instability and conversion to cancer cells. Moreover, it can generate superoxide (O_2^-) and derivative reactive oxygen species (ROS), such as H_20_2 and hydroxyl radical, damaging colonic DNA (Huycke et al., 2002). However, *E. faecalis* is one of the first colonizers of the infants' GIT, in which it plays a protective role by stimulating interleukin (IL)-10 expression and attenuating the expression of proinflammatory cytokine (Wang et al., 2014). On one hand, a protective role has been observed, especially during the early stages of life, on the other hand, it has been defined as a CRC-relevant species. Some authors suggested that different effects could be related to different strains. De Almeida et al. (2019) isolated different *E. faecalis* strains from healthy subjects and CRC patients and observed an antiproliferative role only for the strains isolated from healthy donors. *L. rhamnosus* DBPZ0428 was the most effective strain in the coaggregation with *E. faecalis* (8.6%). *L. paracasei* LMG25880 (6.4%), *L. paracasei* DSM 4905 (6.0%), and *L. rhamnosus* DBPZ0448 (5.3%) also presented a relatively higher coaggregation. Nine strains had a coaggregation between 2.2 and 4.2% and 34 strains,

corresponding to about 50% of the total strains studied, had a coaggregation between 0.0 and 1.9%. The remaining strains (about the 40% of the total) had a negative coaggregation index, ranging between -0.1 and -4.1%. Taheri et al. (2009) did not find coaggregation ability of LAB with enteric pathogens and Jin et al. (1998) detected a coaggregation ability between LAB and *E. coli* lower than 5%. Coaggregation ability does not exhibit a correlation with hydrophobicity and the adhesion ability to SW480 (Table 4.3). However, it has been detected a negative correlation between coaggregation and autoaggregation (ρ=-0.155, p=0.02; Table 4.3) similarly to Collado et al. (2008), although they observed also a positive or any correlation, depending on the strains tested. In this study, the strain with the lower autoaggregation ability was the strain with the higher coaggregation ability. Probably, bacteria cells that do not self-aggregate may be available for coaggregation with pathogens, however, this is only an assumption. Coaggregation with pathogens contributes to the reduction of enterocyte colonization by pathogens and therefore is an important property to be evaluated during the probiotic selection procedure, taking into account that it may vary depending on the pathogens (Li et al., 2020).

Table 4.3 Spearman's correlations between variables.

	Autoaggregation (%)	Hydrophobicity (%)	Coaggregation (%)	Adhesion (%)
Autoaggregation (%)	-			
Hydrophobicity (%)	ρ=0.126 p=0.06	-		
Coaggregation (%)	ρ=-0.155 p=0.02	ρ=-0.085 p=0.209	-	
Adhesion (%)	ρ=-0.315 p<0.01	ρ=-0.178 p<0.01	ρ=-0.118 p=0.08	-

4.3.5 Clustering analysis using heat map and PCA methods

As can be seen in Figure 4.1, the strains tested clustered in distinct groups. Clearly, two main clusters can be observed (I and II). Lactic acid bacteria belonging to the cluster I are characterized by high adhesion ability and low autoaggregation and, conversely, those belonging to the other main cluster (I) are characterized by low adhesion ability and high autoaggregation. This trend is confirmed also by Spearmen's correlation coefficient, which

represents the relationship between adhesion and autoaggregation, which was the strongest detected in this study (ρ=-0.315, $p<0.01$). The strains are more or less equally distributed regarding the number into the two main clusters (I and II). Surprisingly, in the cluster I, in which are present almost all of the strains with the highest adhesion ability, the 78% of the strains were isolated from food products and only the 20% are of human origin (Figure 4.1). Conversely, in the cluster II it can be observed that the 68% and 36% of the strains are of human and food origin, respectively. Conceptually, LAB isolated from humans are well adapted to the GIT environment and therefore are supposed to have a higher probiotic potential compared to other LAB (Gu et al., 2008; Davoodabadi et al., 2015). However, our results are in contrast to those of other studies in which the adhesion ability of LAB isolated from food sources is lower compared to the LAB of human origin (Federici et al., 2014). Some other authors observed and suggested that LAB of human origin not necessarily are related to higher adhesion ability (Monteagudo-Mera et al., 2012). Looking at Figure 4.1, it can be clearly seen the equal distribution of the three species in the two main cluster (I and II). The projection of the different strains based on the principal component analysis (PCA; Figure 4.2) highlighted the diversity among strains belonging to the same species. In total, about 64% of the variation was explained by two principal components analysis (PC1: 36.1%; PC2: 27.97%). As demonstrated by the heatmap (Figure 4.1) and PCA (Figure 4.2) and according to the literature, the cell surface properties tested in this study can be defined as strain-specific and independent from the isolation sources.

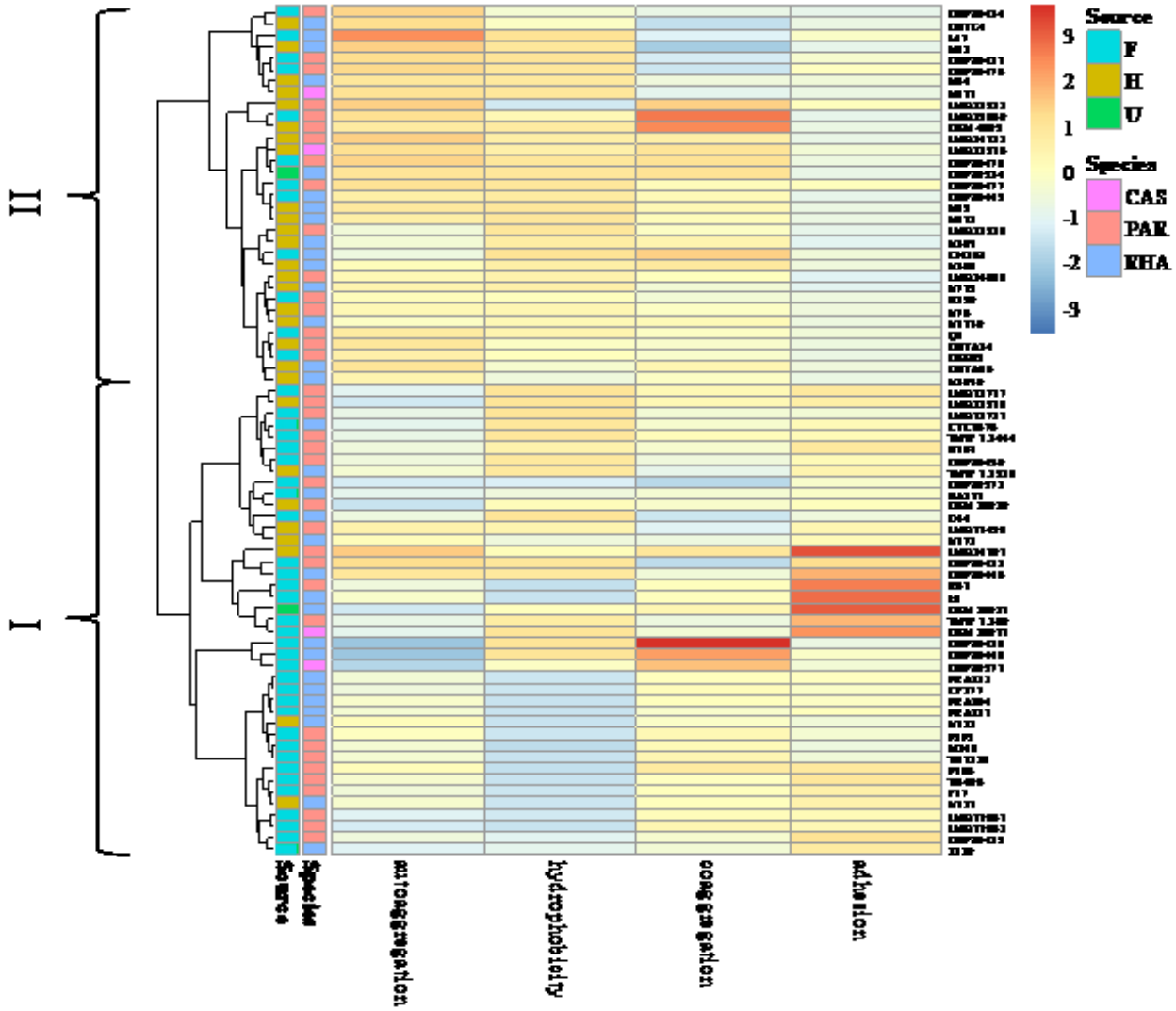

Figure 4.1 Cluster analysis of LAB strains based on their cell surface properties using heat-map method.

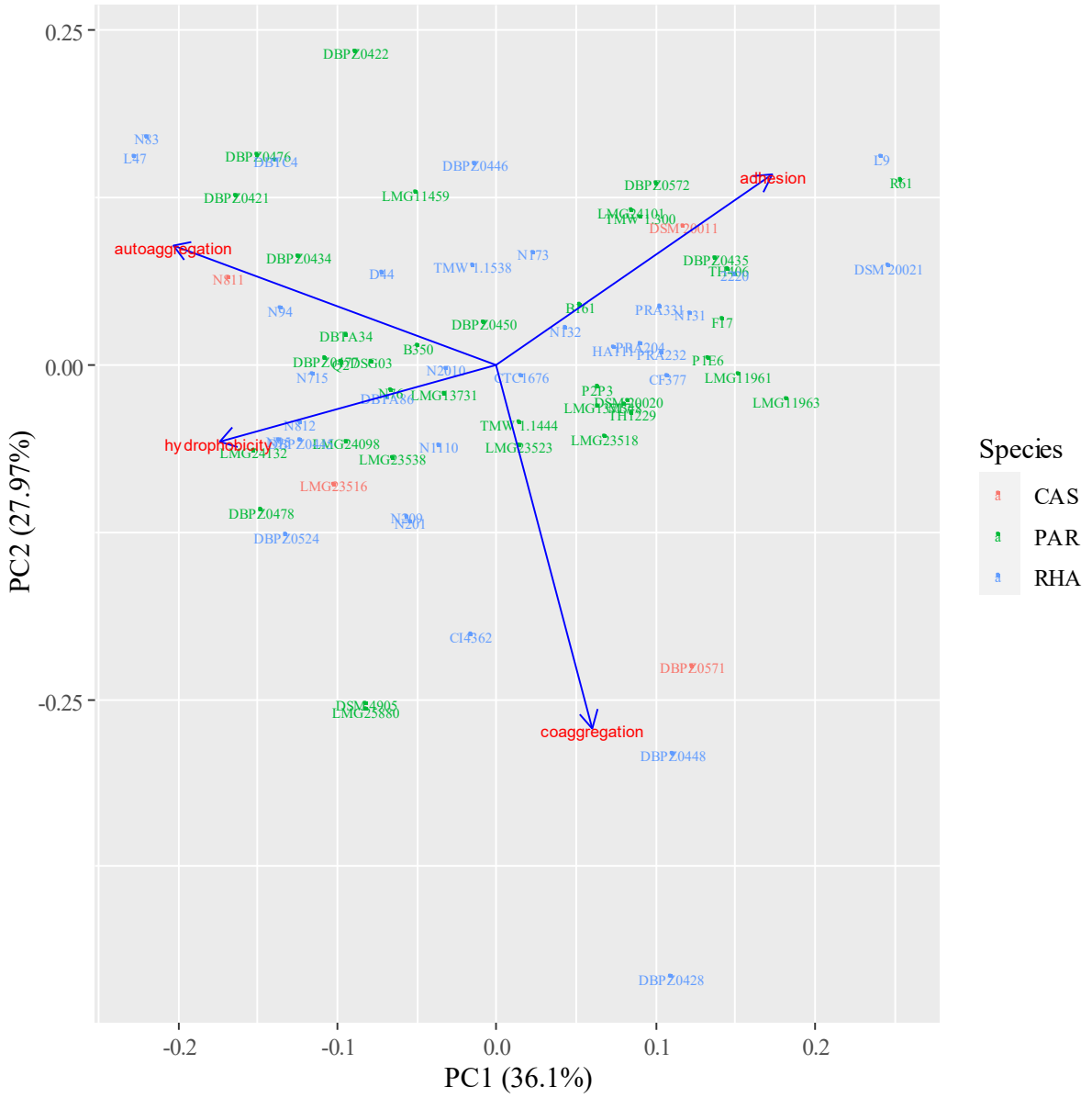

Figure 4.2 Cell surface properties of LAB strains via principal component analysis (PCA).

4.4 Conclusions

Several strains evaluated in this study showed potential to be used as probiotics as they were able to efficiently adhere to intestinal epithelial cells which is essential to play a beneficial role in the intestinal tract. However, future investigations are required to assess their potential benefits to human health and their technological characteristics before their utilization as probiotics in functional foods and supplements. Overall, the tested strains were not able to coaggregate with *E. faecalis*, in future studies, the ability to coaggregate with other pathogens

of food and human origin will be also tested. In literature, some studies suggested that bacteria autoaggregation ability and hydrophobicity are adhesion related properties and therefore they can be used to predict which strains can have a higher adhesion ability. However, in some other studies, the relationship between adhesion and autoaggregation or adhesion and hydrophobicity has presented contrasting results. This study agrees with the latter studies, concluding that using autoaggregation and hydrophobicity as indicators for adhesion ability is not so useful, at least for the tested species. Moreover, the results of this study suggested that the isolation of potential probiotic strains from human sources should not constitute a forced choice, since the strains isolated from food sources were those with the higher adhesion ability, compared to those of human origin.

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5. Heat-killed cells and cell-free supernatants of lactobacilli: screening for antiproliferative effect in human colorectal cancer cells

5.1 Introduction

Although the searching for the so called "Next Generation Probiotics" from the genera *Bacteroides*, *Clostridium*, *Faecalibacterium*, *Akkermansia* or other than those traditionally used, lactic acid bacteria (LAB) and especially lactobacilli, remain the most studied and used probiotics to be included in functional foods and dietary supplements (Saarela, 2019). Probiotics, as it is frequently reiterated, are defined by FAO and WHO as "live microorganisms, which when administered in adequate amounts confer a health benefit on the host". However, recent evidence demonstrate that also dead/inactive cells and the produced metabolites of probiotics could have a positive effect on human health (Bouter et al., 2018; Luceri et al., 2016; Lebeer et al., 2012; Algieri et al., 2023). Consequently, new terminologies in this field has been gradually developed. Parapostbiotics is a term referring to cell envelope components including peptidoglycan, teichoic acids, cell wall polysaccharides, cell surface proteins and other molecules that constitutes the probiotics cell wall, whereas postbiotics include proteins, peptides including bacteriocins, organic acids (short chain fatty acids), conjugated linoleic acid as well as neurotransmitters (γ-aminobutyric acid, serotonin, dopamine, etc.) that can be secreted by live bacteria or released after bacterial lysis (Zendeboodi et al., 2020). All these "probiotics fractions" are gaining more and more interest especially due to the emerging concern regarding safety problems associated with live microbial cells as the possible transmission to pathogens of antibiotic-resistant genes via horizontal gene transfer (Colautti et al., 2022). Once these enter the human gastrointestinal tract (GIT), several health benefits are being exerted, depending on the strain, including anticancer, antimicrobial, and immunomodulatory activities. Some of the beneficial roles of LAB can be exerted as a component of the intestinal microbiota. As it is well known, intestinal microbiota and its composition are linked and implicated in the human health and gut dysbiosis; this latter usually foresees a reduction in bacteria with beneficial potential which

has been associated with the onset of many disorders (Chidambaram et al., 2022; Zhang et al., 2020; Karlsson et al., 2012). Chemotherapy and radiotherapy have several side effects, although they are effectively used for cancer treatment, and therefore the search and interest for novel anticancer treatment alternatives are growing. In particular, drugs derived from natural sources, such as plant and microorganisms are attracting much attention for their efficacy and low adverse effects. The prominent role of LAB for their potential anti-colorectal cancer activity both *in vitro* and animal studies is well discussed in recent reviews (Tripathy et al., 2021; Eslami et al., 2019; Brasiel et al., 2020). It must be underlined that evidence of beneficial effects of probiotics result by *in vitro* studies must be confirmed by *in vivo* studies. Probiotics may activate anticancer mechanisms in the human gut through two main mechanisms: i) prevention of carcinogenesis process or ii) inhibition of cell proliferation and induction of apoptosis in cancer cell. Many studies have evidenced that the composition of the intestinal microbiota of patients with colorectal cancer differs to some extent from those of healthy subjects (Moore & Moore, 1995; Wang et al., 2012). Subsequently, the prevention of cancer may occur through the modification of the intestinal microbiota, restoring the balance of gut microbiota by the increase of the growth of beneficial bacteria and inhibiting harmful bacteria (Song et al., 2018; Liu et al., 2022). Other important mechanisms are the ability of LAB to bind and degrade carcinogenic compounds (Hernandez-Mendoza et al., 2011; Zhao et al., 2013; Rivas-Jimenez et al., 2016) and to modulate the immune system, reducing the intestinal inflammatory status which is believed to promote carcinogenesis (Ullman & Itzkowitz, 2011; Rubin et al., 2012). Besides the role of LAB in the prevention of intestinal cancer, in the last years, the antiproliferative activity of several LAB strains and their cellular components or metabolites have been studied on different cancer cell lines including those coming from bladder, breast, cervical, gastric, and colorectal carcinoma (Pourbaferani et al., 2021; Nada et al., 2020; Pawar & Aranha, 2022; Mantzourani et al., 2019; Maghsood et al., 2020; Ren et al., 2020). In this study, the antiproliferative activity of heat-killed cells and cell-free supernatants obtained from 60 strains belonging to *Lacticaseibacillus* species were tested *in vitro* on the human colorectal cancer cell line SW480.

5.2 Materials and methods

5.2.1 Bacterial strains and growth conditions

A total of 60 strains (Table 5.1) were assessed for their antiproliferative activity. The strains were stored at -80 °C as 30% (vol/vol) glycerol stock-cultures in de Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Milan, Italy) and routinely propagated (1% w/v) in MRS broth for 24 h at 37 °C before the analysis.

Table 5.1 *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei* and *Lacticaseibacillus rhamnosus* strains used in this study and their relative isolation sources.

Origin	Identification
Raw milk	*L. paracasei*: P1E6[3], P2P3[3] *L. rhamnosus*: HA111[4]
Cheese	*L. casei*: DSM20011[1] *L. paracasei*: LMG25880[1], DBPZ0421[8], DBPZ0422[8] DBPZ0434[8], DBPZ0435[8], DBPZ0476[8], DBPZ0477[8,] DBPZ0478[8], M348[8], DSG03[8], TH1229[9], TH406[9], TMW1.1444[6], TMW1.1259[6], R61[13], F17[13] *L. rhamnosus*: PRA204[5], PRA232[5], PRA331[5], DBPZ0428[8], DBPZ0445[8], DBPZ0448[8], CI4362[12], CF377[12], D44[13], 5D9T[9], L9[9], L47[9]
Fermented sausages	*L. rhamnosus*: CTC1676[14], 2220[15]
Sourdough	*L. paracasei*: DBPZ0572[8], Q2[8] *L. casei*: DBPZ0571[8]
Wine, must, and cellar equipment's	*L. paracasei*: LMG11961[1], LMG11963[1], LMG13717[1] LMG13731[1], B161[11], B350[11]
Elisir	*L. paracasei*: LACcas29[7]
Coffee	*L. casei*: DSM20178[2]
Human	*L. casei*: LMG23516[1] *L. paracasei*: DSM20020[2], LMG11459[1], LMG23518[1], LMG23523[1], LMG24132[1], DSM4905[2] *L. rhamnosus*: DBTC4[10], N94[16], N95[16], N83[16], N812[16], N1110[16], TMW1.1538[6]
Unknown	*L. rhamnosus*: DSMZ20021[2]

[1]LMG: BCCM/LMG, Belgian Co-ordinated Collections of Micro-organisms (BCCM™), Belgium.
[2]DSM: DSM, Deutsche Sämmlung von Mikroorganismen und Zellkülturen, Braunschweig, Germany
[3]Dipartimento di Agraria, Università degli Studi di Sassari, Sassari, Italy.
[4]Harmonium International Inc., Mirabel, Canada.
[5]Dipartimento di Scienze Agrarie e degli Alimenti, Università delgi Studi di Modena e Reggio Emilia, Italy
[6]Lehrstuhl für Technische Mikrobiologie, Technische Universität München, Freising, Germany
[7]Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università degli Studi di Milano, Italy
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[9]Veneto Agricoltura, Istituto per la Qualità e le Tecnologie Agroalimentari, Thiene (VI), Italy
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5.2.2 SW480 cell culture

The human colorectal adenocarcinoma cell line SW480 was kindly provided by the Department of Medicine of the University of Udine (Italy). The cell line was cultured in Dulbecco's Modified Eagles Medium (Stable cell DMEM; Sigma-Aldrich, Milan, Italy) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Milan, Italy) and maintained in 25 cm^2 or 75 cm^2 culture flasks (Sarstedt, Nümbrecht, Germany) in a humidified-atmosphere with 5% CO_2 and 95 % air in an incubator (Euroclone, Milan, Italy) at 37 °C. Every two days confluent cultures were split 1:3, after washing with phosphate buffered saline (PBS; pH 7.4, Sigma-Aldrich, Milan,) using trypsine-EDTA (SAFC Biosciences, Lenexa, KS, USA).

5.2.3 Preparation of cell-free supernatants (CFS) and heat-killed cells (HK cells)

Lactic acid bacteria were cultured in MRS broth under static conditions at 37 °C for 24 h. Broth cultured bacterial cells were centrifuged at 9,700 x g for 10 min. Supernatants were collected and filtered through 0.2 μm Minisart filters (Sartorius Biotech, Göttingen, Germany). Part of the CFS was buffered with 1 M NaOH solution in order to obtain a neutralized cell-free supernatant (CFSn) with a pH value of 7.8 ± 0.2. Pellets obtained from the centrifugation of the broth cultures were washed twice with phosphate-buffered saline (PBS) and resuspended in the same buffer. Absorbance at an optical density of wavelength of 600 nm (A_{600}) was adjusted to 0.10 ± 0.01 to standardize the bacteria concentration (10^7 CFU/ml). Bacterial suspensions were heated at 95 °C for 20 min. To ensure the inactivation of lactic acid bacteria, the suspension was subjected to plate count on De Man, Rogosa, and Sharp (MRS, Oxoid, Italy) agar and incubated at 37 °C for 48 h. In order to verify also the presence of survived viable but not culturable (VBNC) cells, the suspensions were subjected to double enrichment culture in MRS broth for 48 h at 37 °C. The presence of reactivated VBNC cells was then verified by isolation on MRS agar. Heat-killed cells were centrifuged at 9,700 x g for 10 min and the pellets were resuspended in the same volume of DMEM prior to application onto the SW480 cells.

5.2.4 MTT assay

MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide) assay is the most common used method for high throughput screening of cytotoxic properties of compounds on cell lines. This assay quantifies the amount of formazan produced from MTT reduction by the mitochondrial activity that is directly proportional to the number of viable cells. The cytotoxic effect of HK cells, CFS and CFSn were assessed on human colorectal adenocarcinoma cell line SW480. SW480 cells were seeded onto 96-well tissue culture plates (Sarstedt, Nümbrecht, Germany) at a concentration of 3.0 x10^4 cells/well in 200 µL of DMEM and incubated for 24 h. The cells were then subjected to the activity of i) the CFS, ii) the CFSn, and iii) the HK cells. In particular, the culture medium was replaced with 100 µL of fresh medium and the cells were then incubated with 100 µL of CFS (i) for 24 h, CFSn (ii) for 24 and 48 h, and 100 µL of HK cells at two different concentrations (10^7 CFU/mL and 10^6 CFU/µL) for 24 h. The incubation was performed at 37 °C in a humidified atmosphere with 5 % CO_2 and 95 % air for all the different trials. Cells incubated with 200 µL of DMEM were used as a control. After the incubation period, cells were washed with 200 µL of PBS and treated with 40 µL of MTT solution (0.5 mg/mL in PBS; Sigma-Aldrich, Milan, Italy) and incubated in the dark for 3 h in a humidified atmosphere of 5 % CO_2 95 % air at 37 °C. The formazan crystals formed were solubilized by the addition of 150 µL/well of acidic isopropanol (0.04 M HCl-isopropanol). Absorbance was measure at OD_{570} using a Sunrise microplate reader (Tecan, Milan, Italy) and cell viability was determined according to the following equation:

$$Cell\ viability\ (\%) = \frac{treatment\ OD_{570nm} \times 100}{control\ OD_{570nm}}$$

5.2.5 Statistical analysis

Results obtained from MTT assay were analyzed with one-way analysis of variance (ANOVA) and Tukey's post hoc analysis at the ($p<0.01$) significance level. The statistical analysis was performed using R software version 4.2.2.

5.3 Results and discussion

5.3.1 Preparation of cell-free supernatants (CFS) and heat-killed cells (HK cells)

CFS were checked for the absence of LAB cells by both microbiological analysis and microscopic evaluation. Results demonstrated the purity of the supernatants, confirming the absence of microrganisms. Regarding the evaluation of the effectiveness of the killing treatement of the microbial cells, the plate count analysis showed that the samples resulted < 10 CFU/mL, and the following enrichment test demonstrated the absence of viable LAB cells in the samples.

5.3.2 Antiproliferative effect in human colorectal cancer cells

The ability of probiotics to control and modulate cancer cells proliferation and apoptosis are of great importance and the research on the prevention and treatment of cancer have involved this class of microorganisms. In this study, 60 strains belonging to *L. casei*, *L. paracasei*, and *L. rhamnosus* (Table 1) were investigated for their antiproliferative activity against human colorectal cancer cell line SW480. The use of death cells or metabolites of probiotics may avoid some risks related to the use of live cells (Kataria et al., 2009). MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide) assay was chosen to measure the cell viability of SW480 after the aforementioned treatments because it resulted the most common used method for high throughput screening of cytotoxic properties of compounds on cell lines. This assay quantifies the amount of formazan produced from MTT reduction by the mitochondrial activity that is directly proportional to the number of viable cells (Stepanenko & Dmitrenko, 2015). The treatment with HK cells at any concentration did not have any effect on the cell viability of SW480 (Table S5.1; $p>0.01$). Botta et al. (2022) investigated the effect on colon cancer cell of both viable and death of *Lactiplantibacillus plantarum* probiotics cells. On the contrary to the viable bacteria, HK cells did not have any effect on cell viability, according to the results of the present study, suggesting that the use of high temperature to kill LAB cells can have inactivated some heat-sensitive cell wall components involved in the anti-cancer activity. However, in other studies, HK cells have produced a considerable decrease on cancer cell viability, similarly to that of the viable cells (Orlando et al., 2012; Karimi Ardestani et al., 2019; Choi et al., 2006). Different studies have associated beneficial activity to the extracellular metabolites produced by probiotics. In particular, the induction of

apoptosis was associated to butyrate, produced in the colonic lumen by the bacterial fermentation of dietary fiber (Zeng et al., 2020). Giving this evidence, the interest in butyrate producing-bacteria and the search for strategies to induce the "butyrogenic effect" are growing. Other short chain fatty acids (SCFAs), such as acetate and propionate are resulted to be involved in producing beneficial effects to human health (Mirzaei et al., 2021). Anti-cancer activity of *Enterococcus faecalis* CFS was related to proteinaceous compounds, since apoptosis induction were lost after subjection of the CFS to proteolytic enzymes (Salek et al., 2023). Exopolysaccharides (EPS) are other molecules that are gaining interest for their multifaceted activity on food production and human health. Some studies have demonstrated the antiproliferative activity of EPS produced by various LAB such as *L. casei*, *Lactobacillus acidophilus*, and *Lactobacillus helveticus* (Liu et al., 2011; Deepak et al., 2016; Xiao et al., 2020). In Table 5.2 were shown the SW480 cell viability values obtained using CFS and neutralized CFS (CFSn). The results demonstrated that cell viability of the treated cells was significantly different from the untreated cells ($p<0.01$). The use of CFS without pH adjustment, produced after 24 h, an almost total inhibition of the SW480 cell viability. In fact, the cell viability after 24 of treatment with CFS ranged from 0.51% to 6.83% (Table 5.2). Each LAB strain used in this study was grown for 24 h at 37 °C in MRS broth. The decrease of the medium pH after LAB growth is well known. The pH of the MRS after 24 h ranged from 3.68 ± 0.01 to 4.55 ± 0.02. At a first deduction, the antiproliferative activity of CFS, considering their pH, could be related to the inappropriate pH for the growth of this cell line, since a neutral pH is considered to be optimal. For this reason, in this study the pH of CFSs was neutralized to investigate if the antiproliferative activity was related to the improper growth conditions of the cell lines or if other bioactive molecules may be involved (Table 5.2). Surprisingly, a significative reduction in cell viability compared to the untreated cells was obtained also using the CFSn ($p<0.01$), which showed a cell viability reduction between 12.59% and 83.72% (Table 5.2). However, for the 80% of the CFSn tested, the reduction of cell viability after 24 h of treatment was significantly lower compared to that obtained with CFS ($p<0.01$). According to our results, other authors have detected a lower antiproliferative activity of the neutralized CFS (Jafari-Nasab at al., 2021; Khaleghi et al., 2023). Regarding the remaining 20 %, the viability of the cells treated with CFS was not significantly different from that treated with CFSn ($p>0.01$).

Table 5.2 Cell viability (%) of SW480 after the treatment with probiotics cell-free supernatants (CFS) for 24 h and neutralized cell-free supernatants (CFSn) for 24 and 48 h.

Strain	Cell viability (%)		
	CFS (24 h)	CFSn (24 h)	CFSn (48 h)
HA111	$2.72 \pm 0.84^{ghijklmnopA}$	31.58 ± 22.69^{cA}	0.35 ± 0.23^{fA}
LMG23518	$2.40 \pm 0.38^{hijklmnopB}$	30.53 ± 2.70^{cA}	4.62 ± 0.92^{defB}
F17	$2.74 \pm 0.29^{ghijklmnopB}$	19.77 ± 7.09^{cA}	1.42 ± 1.02^{defB}
D44	$3.04 \pm 0.83^{fghijklmnoA}$	30.74 ± 18.71^{cA}	1.27 ± 0.23^{defA}
DBPZ0478	1.88 ± 0.08^{lmnopB}	65.32 ± 28.30^{abcA}	33.75 ± 53.71^{defB}
DBPZ0434	$2.36 \pm 0.27^{hijklmnopA}$	46.38 ± 32.70^{abcA}	2.58 ± 0.27^{defA}
DBPZ0445	5.45 ± 0.22^{abcdeB}	16.28 ± 1.88^{cA}	1.98 ± 1.72^{defB}
DSG03	1.98 ± 0.33^{klmnopB}	26.75 ± 5.27^{cA}	4.11 ± 2.45^{defB}
N94	$2.41 \pm 0.13^{hijklmnopB}$	23.76 ± 9.23^{cA}	3.86 ± 2.74^{defB}
N83	1.46 ± 0.08^{nopB}	64.02 ± 29.78^{abcA}	4.56 ± 0.92^{defB}
LMG11459	1.75 ± 0.97^{lmnopC}	38.46 ± 4.75^{abcA}	30.94 ± 1.82^{bB}
N1110	$4.34 \pm 0.65^{bcdefghijkB}$	31.84 ± 6.59^{cA}	1.62 ± 0.70^{defB}
DBPZ0428	$2.24 \pm 0.26^{hijklmnopB}$	59.80 ± 7.96^{abcA}	8.37 ± 2.25^{cdefB}
DBPZ0421	$2.15 \pm 0.31^{ijklmnopB}$	63.28 ± 26.11^{abcA}	4.92 ± 3.39^{defB}
DBPZ0571	$3.68 \pm 0.26^{defghijklmnB}$	42.68 ± 16.76^{abcA}	1.12 ± 0.23^{efB}
DBPZ0422	$3.59 \pm 0.33^{defghijklmnB}$	43.26 ± 5.35^{abcA}	4.92 ± 0.93^{defB}
DBPZ0435	4.96 ± 1.94^{abcdefgB}	34.24 ± 3.23^{abcA}	3.29 ± 3.49^{defB}
N95	1.81 ± 0.23^{lmnopB}	53.93 ± 6.38^{abcA}	9.19 ± 3.92^{cdefB}
LMG23523	$2.83 \pm 0^{fghijklmnopA}$	50.16 ± 29.69^{abcA}	3.61 ± 2.33^{defA}
DSM20178	$2.49 \pm 0.32^{hijklmnopB}$	32.86 ± 3.77^{cA}	4.85 ± 1.25^{defB}
DBPZ0448	$2.50 \pm 0.20^{hijklmnopB}$	23.76 ± 4.07^{cA}	1.97 ± 0.77^{defB}
LMG24132	$3.49 \pm 0.59^{defghijklmnA}$	46.74 ± 35.10^{abcA}	4 ± 1.38^{defA}
L47	6.83 ± 0.43^{aB}	87.41 ± 11.77^{aA}	9.02 ± 1.52^{cdefB}
DBTC4	$4.47 \pm 0.14^{abcdefghijB}$	41.51 ± 17.23^{abcA}	2.25 ± 0.68^{defB}
N812	5.18 ± 0.31^{abcdefA}	32.02 ± 17.89^{cA}	3.72 ± 2.35^{defA}
B350	5.73 ± 0.68^{abcdAA}	25.95 ± 15.07^{cA}	2.08 ± 0.83^{defA}
Q2	5.69 ± 0.34^{abcdA}	43.9 ± 37.57^{abcA}	4.28 ± 2.25^{defA}
DSM4905	$2.87 \pm 0.32^{fghijklmnopB}$	42.79 ± 5.89^{abcA}	1.86 ± 1.18^{defB}
LMG23516	1.79 ± 0.46^{lmnopB}	41.55 ± 4.7^{abcA}	0.9 ± 0.19^{efB}
DBPZ0477	1.53 ± 0.16^{lmnopB}	59.36 ± 7.85^{abcA}	6.7 ± 1.03^{defB}
DBPZ0476	1.4 ± 0.28^{nopC}	66.84 ± 4.44^{abcB}	8.68 ± 1.20^{cdefB}
LMG11963	$4.09 \pm 0.60^{bcdefghijklB}$	36.4 ± 0.95^{abcA}	1.06 ± 0.10^{efC}
CF377	6.38 ± 0.46^{abB}	30.30 ± 5.11^{cA}	2.59 ± 0.52^{defB}
LMG13731	1.56 ± 0.29^{lmnopC}	42.79 ± 3.27^{abcA}	8.84 ± 1.61^{cdefB}
2220	$3.07 \pm 1.61^{efghijklmno\ B}$	25.79 ± 0.91^{cA}	1.18 ± 0.51^{efB}
DSM20020	0.90 ± 0.35^{opA}	45.92 ± 26.98^{abcA}	4.09 ± 0.76^{defA}
DSM20021	1.7 ± 0.24^{lmnopB}	23.25 ± 2.33^{cA}	4.09 ± 1.43^{defB}
DSM20011	1.79 ± 0.29^{lmnopB}	28.63 ± 1.47^{cA}	2.00 ± 0.73^{defB}
P2P3	$2.26 \pm 1.25^{hijklmnopB}$	21.78 ± 3.89^{cA}	5.54 ± 2.06^{defB}
PRA204	1.65 ± 0.34^{mnopA}	30.42 ± 20.19^{cA}	4.2 ± 1.50^{defA}

L9	1.89 ± 0.54^{lmnopB}	47.72 ± 20.11^{abcA}	9.85 ± 9.28^{cdefB}
M348	0.51 ± 0.38^{pC}	23.41 ± 2.35^{cA}	7.27 ± 1.99^{cdefB}
LMG13717	$2.37 \pm 0.08^{hijklmnopB}$	33.2 ± 7.81^{bcA}	3.97 ± 1.28^{defB}
R61	1.85 ± 0.08^{lmnopB}	18.76 ± 3.12^{cA}	3.47 ± 0.86^{defB}
CTC1676	$3.23 \pm 2.27^{efghijklmnoB}$	53.37 ± 8.56^{abcA}	3.19 ± 0.60^{defB}
LMG11961	1.64 ± 0.46^{lmnopB}	56.51 ± 15.51^{abcA}	5.09 ± 0.35^{defB}
CI4362	1.83 ± 0.63^{lmnopB}	45.41 ± 18.99^{abcA}	11.81 ± 1.89^{cdefB}
LMG25880	$4.58 \pm 0.21^{abcdefghA}$	37.83 ± 21.50^{abcA}	4.08 ± 0.39^{defA}
TMW1.1259	$2.24 \pm 1.51^{hijklmnopB}$	86.29 ± 5.4^{abA}	2.45 ± 0.09^{defB}
TH406	1.49 ± 0.30^{nopB}	42.42 ± 2.32^{abcA}	4.083 ± 0.98^{defB}
P1E6	2.04 ± 0.31^{klmnopA}	54.75 ± 41.80^{abcA}	13.52 ± 4.53^{cdA}
5D9T	1.94 ± 0.15^{lmnopB}	29.25 ± 6.15^{cA}	19.31 ± 5.10^{bcA}
PRA331	1.50 ± 0.39^{nopA}	21.29 ± 11.96^{cA}	$4.95 \pm 1.^{37defAA}$
PRA232	$3.89 \pm 1.08^{cdefghijklmB}$	36.92 ± 14.53^{abcA}	10.26 ± 2.41^{cdefB}
DBPZ0572	6.21 ± 1.37^{abcB}	31.74 ± 1.60^{cA}	7.12 ± 1.51^{cdefB}
B161	6.07 ± 0.98^{abcB}	30.24 ± 3.00^{cA}	5.67 ± 1.17^{defB}
TMW1.1444	$3.25 \pm 1.21^{efghijklmnoB}$	29.77 ± 3.36^{cA}	4.22 ± 0.21^{defB}
TH1229	$2.27 \pm 0.54^{hijklmnopB}$	35.98 ± 2.84^{abcA}	13.15 ± 4.25^{cdeA}
LACcas29	2.09 ± 0.26^{jklmnopB}	36.69 ± 8.74^{abcA}	51.78 ± 21.85^{aA}
TMW1.1538	$4.09 \pm 0.60^{bcdefghijklB}$	37.63 ± 3.55^{abcA}	9.42 ± 4.39^{cdefB}

Different lowercase letters (a-p) indicate significant differences ($p<0.01$) among the same column. Different uppercase letters (A-C) indicate significant differences among the same row.

On the contrary, the treatment of SW480 with the 87 % of CFSn for 48 h produced a reduction in cell viability similar to CFS. However, the antiproliferative activity may probably be related to different mechanisms. As it can be seen in Table 5.2, the increase of the treatment time of SW480, from 24 h to 48 h, have produced a significant decrease of the SW480 cell viability using the 70 % of CFSn ($p<0.01$). The remaining 30 % produced a similar reduction in viability obtained with the treatment with CFSn for 24 h ($p>0.01$). Time-dependent antiproliferative activity were observed also in other studies (Tiptiri-Kourpeti et al., 2016; Botta et al., 2022). The cell viability reduction of 50% is usually adopted to define a compound cytotoxic. The tested CFS and CFSn applied for 24 and 48 h, produced a reduction in cell viability lower than 50%, except after the 24 h treatment with the CFSn of 13 strains. Overall, it can be stated that the metabolites contained in the CFS produced by all the strains of the 3 species analyzed in this study have a considerable antiproliferative activity potential against human colorectal cancer cell, although it will be necessary to assess their activity using other colorectal cancer cell lines as the results may be different (Gonzalez-Sarrias et al., 2014).

5.4 Conclusions

In this study, the HK cells and CFS derived from probiotic strains belonging to *L. casei*, *L. paracasei* and *L. rhamnosus* species, isolated from different sources, were tested against the SW480 human colorectal cancer cell line. All the tested CFS produced a significative reduction on SW480 cell viability. Death by necrosis, compared to death by apoptosis (programmed cell death), may damage the neighbor healthy cells and causing inflammation. Consequently, finding and using compounds able to induce apoptosis may be an effective technique in the treatment of cancer. Therefore, in future studies will be fundamental understanding the mechanisms of cell death after the CFS treatment and evaluate the content of the supernatants. Future studies will be performed to individuate which metabolite/s is/are responsible for the antiproliferative effect and to understanding the mechanism involved in the reduction of cell viability.

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Supplementary materials

Table S5.1 Cell viability (%) of SW480 after 24 h of treatment with heat-killed (HK) cells at different concentrations of probiotic strains.

Strain	10^7 CFU/mL	10^6 CFU/mL
HA111	80.80 ± 4.20	88.40 ± 7.53
LMG23518	92.27 ± 4.88	89.89 ± 5.82
F17	85.48 ± 9.93	92.06 ± 9.58
D44	82.97 ± 8.36	86.02 ± 12.55
DBPZ0478	78.94 ± 7.36	82.06 ± 1.64
DBPZ0434	81.82 ± 7.25	84.88 ± 4.51
DBPZ0445	84.0 ± 3.39	93.88 ± 4.78
DSG03	85.52 ± 14.53	92.07 ± 11.93
N94	82.69 ± 13.80	82.64 ± 10.76
N83	93.54 ± 7.69	93.70 ± 5.71
LMG11459	79.18 ± 5.32	87.18 ± 10.42
N1110	89.86 ± 7.96	92.53 ± 13.70
DBPZ0428	85.01 ± 4.50	85.21 ± 3.81
DBPZ0421	93.33 ± 5.60	87.24 ± 12.17
DBPZ0571	84.60 ± 1.96	80.52 ± 7.44
DBPZ0422	85.60 ± 2.26	80.83 ± 6.91
DBPZ0435	88.69 ± 8.91	90.75 ± 9.64
N95	85.69 ±3.51	96.24 ± 8.07
LMG23523	77.72 ± 4.55	79.18 ± 9.41
DSM20178	83.18 ± 10.50	86.23 ± 8.08
DBPZ0448	79.18 ± 9.41	89.26 ± 2.32
LMG24132	91.11 ± 5.40	89.28 ± 9.41
L47	91.66 ± 3.56	91.33 ± 5.95
DBTC4	95.08 ± 6.10	91.82 ± 8.07
N812	86.65 ± 13.20	89.61 ± 9.95
B350	91.11 ± 5.40	89.42 ± 2.43
Q2	81.60 ± 8.54	92.62 ± 6.23
DSM4905	81.00 ± 5.22	83.31 ± 3.64
LMG23516	86.50 ± 4.43	91.92 ± 7.14
DBPZ0477	80.81 ± 1.95	85.89 ± 14.95
DBPZ0476	82.86 ± 4.36	89.47 ± 7.21
LMG11963	92.27 ± 4.88	89.56 ± 6.26
CF377	89.08 ± 4.41	94.06 ± 6.21
LMG13731	84.51 ± 6.99	92.69 ± 8.51
2220	78.90 ± 7.24	82.10 ± 1.70
DSM20020	83.00 ± 5.22	85.75 ± 4.02
DSM20021	85.01 ± 2.75	93.72 ± 4.14
DSM20011	88.56 ± 10.24	91.74 ± 11.57
P2P3	83.69 ± 12.83	83.64 ± 9.41

PRA204	93.07 ± 7.58	92.71 ± 6.07
L9	78.95 ± 5.89	86.56 ± 10.06
M348	90.46 ± 3.20	91.98 ± 13.82
LMG13717	85.69 ± 3.59	88.31 ± 3.92
R61	92.18 ± 7.00	90.76 ± 8.58
CTC1676	84.06 ± 1.76	84.52 ± 3.33
LMG11961	88.94 ± 6.20	80.78 ± 6.87
CI4362	91.49 ± 6.26	94.08 ± 4.22
LMG25880	81.27 ± 8.2	86.82 ± 3.85
TMW1.1259	77.63 ± 3.98	78.64 ± 8.79
TH406	82.79 ± 11.55	89.41 ± 7.78
P1E6	82.16 ± 3.08	87.92 ± 6.92
5D9T	85.11 ± 9.67	91.78 ± 10.17
PRA331	79.03 ± 7.20	88.73 ± 4.27
PRA232	86.70 ± 13.07	93.89 ± 4.79
DBPZ0572	75.03 ± 1.59	93.96 ± 7.21
B161	92.12 ± 5.99	90.96 ± 4.42
TMW1.1444	82.25 ± 2.43	93.96 ± 7.21
TH1229	81.49 ± 10.22	95.7 ± 11.36
LACcas29	77.40 ± 4.14	95.30 ± 1.24
TMW1.1538	86.87 ± 3.74	87.29 ± 3.07

6. Isolation and characterization of extracellular vesicles from *Lacticaseibacillus rhamnosus* and *Lacticaseibacillus paracasei*

6.1 Introduction

The production of extracellular vesicles (EVs) is considered to be a widespread phenomenon among the three domains of life: Archaea, Bacteria, and Eukaria. Extracellular vesicles are a heterogeneous group of cell-derived spherical lipid bilayer-enclosed vesicles, containing different cargo including DNA, RNA, miRNA, proteins, and lipids (Tkach & Théry, 2016). Extracellular vesicles is a generic term that refers to all the secreted membrane vesicles with different size, structure, and composition, however, they have been classified into subgroups based on their origin and biogenesis. Extracellular vesicles produced by eukaryotic cells can be divided into: i) exosomes, generated by the fusion of multivesicular endosomes with the plasma membrane followed by the release of intraluminal vesicles; ii) ectosome or microvesicles, produced by the outward budding of the plasma membrane; iii) apoptotic bodies, originating by programmed cell death (Van Niel et al., 2018). Regarding bacterial EVs, those produced by Gram-negative bacteria were the first studied and have been termed outer membrane vesicles (OMVs) as they are produced by controlled blebbing of the outer membrane. Nowadays, most of the knowledge regarding bacterial EVs comes from studies performed on Gram-negative pathogens, which have outlined the contribution of EVs in bacterial pathogenesis, due to the delivery of toxins or virulence factors (Kim et al., 2015; Toyofuku et al., 2023). Gram-positive EVs are called membrane vesicles (MVs) and differ from those produced by Gram-negative for their biogenesis, which currently is poorly studied and not yet completely understood, and for their composition, for instance, they lack lipopolysaccharide (LPS) (Liu et al., 2018). Bacterial EVs are implicated in stress response, survival, intercellular competition, lateral gene transfer, and pathogenicity (Bose et al., 2020). An additional biological function is the cell-to-cell communication between bacteria, aimed at the regulation of several biological processes including quorum sensing, biofilm formation, survival, competition, and material exchange. However, it has been demonstrated also their

role in inter-kingdom communications through bacteria-host interactions, catching the attention of several researchers who are currently investigating the possible application of EVs in the diagnosis and treatment of diseases using them as biomarkers and therapeutic carriers (Huang et al., 2023). EVs can interact with the cells of the immune system, affecting the innate and adaptive immune responses. Moreover, it has been observed that they can interact with endocrine and nervous systems (Haas-Neill & Forsythe, 2020). The bacteria-host interaction is supposed to be achieved by the delivery of DNA, RNA, proteins, lipids, and other bioactive molecules. Internalization of EVs in eukaryotic cells has been demonstrated using fluorescent labeling (Seo et al., 2018; Rai & Johnson, 2019). It is an active process and may occur through different routes and mechanisms, but the main mechanisms have been proposed to be endocytosis, followed by lipid rafts internalization and plasma membrane fusion (Ñahui Palomino et al., 2021). The functional effect of EVs does not always require internalization, receptor-ligand interactions on the cell surface may be sufficient to produce some effects in recipient cells (Mulcahy et al., 2014). The role of probiotic-derived EVs has been reported by different authors. The modulation of host immune responses against pathogens exerted by probiotics may, in some cases, occur using EVs as mediators. Studies revealed that probiotic EVs may be involved in the activation of apoptosis and cancer cell death. *Lactobacillus rhamnosus* GG EVs had an antiproliferative effect on hepatic cancer cells by increasing the *bax/bcl-2* expression ratio as a result of the downregulation of *bcl-2* and upregulation of *bax* genes expression (Behzadi et al., 2017). EVs produced by *Lactobacillus kefir*, *Lactobacillus kefiranofaciens*, and *Lactobacillus kefirgranum* kefir-derived inhibited interleukin (IL)-8 expression in the large intestine cell, known as one of the major factors that stimulate the inflammatory pathogenesis of inflammatory bowel disease (IBD) (Seo et al., 2018; Grimm et al., 1996). *Lactobacillus plantarum* EVs treatment led to the upregulation of host defense gene expression contributing to the protection against vancomycin-resistant *Enterococcus faecium* infection, opening an additional and alternative door for the treatment of antibiotic-resistant pathogens (Li et al., 2017). Two functional proteins, P40 and P75, associated with the external surface of *Lactobacillus casei* BL23 EVs have been shown to have protective effects on the intestinal epithelium by increasing nuclear factor κB (NF-κB) levels and inducing the phosphorylation of epidermal growth factor receptor (EGFR) (Bäuerl et al., 2020). *Lactobacillus plantarum* Q7 EVs improved dextran

sulfate sodium (DSS)-induced ulcerative colitis symptoms by reducing the level of DSS-upregulated proinflammatory cytokines (IL-6, IL-1β, IL-2, and tumor necrosis factor [TNF]-α) and regulating the gut microbiota. In particular, pro-inflammatory bacteria (proteobacteria) were reduced and the anti-inflammatory bacteria (Bifidobacteria and *Muribaculaceae*) were increased (Hao et al., 2021). Similar results were obtained with EVs produced by *L. rhamnosus* GG, which decreased the expression of pro-inflammatory cytokines at the gene and protein levels in DSS-induced colitis mice (Tong et al., 2021). Little attention has been paid also to the possibility of using EVs as antimicrobials to improve the safety and quality of food products. Lee et al. (2021) investigated the effect of *L. plantarum* EVs as a novel antimicrobial agent for improving the quality and safety of tuna fish. In this study, tuna fish was coated with EVs and this has led to a decrease in the level of certain quality indices including total volatile base nitrogen (TVB-N), peroxide value (PV), malondialdehyde (MDA), and total bacterial population, demonstrating the potential use of EVs in food preservation. Different authors proposed that EVs are able to repeat some of the functions of the parental bacteria. Al-Nedawi et al. (2015) observed that EVs produced by *L. rhamnosus* JB-1 closely replicated the functional effects of the bacterial cells. In the large intestine, bacterial cells are separated from the epithelial cells by a mucus layer and very few bacteria are directly in touch with the epithelium. Therefore, the communication between the host and commensal bacteria is supposed to occur without a direct process and as mentioned before, EVs may play a mediator role in this regard. It has been observed that EVs are constantly produced at all stages of bacterial growth, however, their amount, content, biogenesis, and specific functional role can differ based on various environmental and host-derived factors. The most physiologically relevant conditions that can produce the aforementioned variations are temperature, growth phase, cell culture composition, iron and oxygen availability, biofilm and planktonic lifestyle, exposure to stress conditions, and more (Burnouf et al., 2019; Orench-Rivera & Kuehn, 2016; Toyofuku et al., 2014). As evidenced, EVs have great potential in different fields, however, their biogenesis, internalization, and composition need to be further investigated, because very little is known. In this study, MVs produced from *Lacticaseibacillus paracasei* and *L. rhamnosus* were isolated and characterized.

6.2 Materials and methods

6.2.1 Strains

Two type strain, *L. paracasei* DSM (Deutsche Sämmlung von Mikroorganismen und Zellkülturen, Braunschweig, Germany) 20020 and *L. rhamnosus* DSM 20021, were choosen to investigate the MVs production and characterization. The strains were stored at -80 °C as 30% (vol/vol) glycerol stock-cultures in de Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Milan, Italy).

6.2.2 Isolation of MVs

Membrane vesicles were isolated using the method described by Baüerl et al. (2020) with some modifications. After 48 h of growth in MRS agar, one single colony of each strain was picked from the MRS agar plates and transferred to MRS broth. Each strain was then propagated almost 3 times as follows: 0.1 mL of bacterial culture was inoculated in 4 mL of fresh MRS broth and incubated for 24 h at 37 °C. Four milliliters of each strain were then used to inoculate 2 L of MRS broth and the culture was grown for 48 h at 37 °C and then centrifuged at 10,000 x *g* for 20 min at 4 °C in 450 mL bottles. Then, the culture supernatant was filtered through a 0.45 μm vacuum filter and 0.2 μm vacuum filter to remove the residual bacteria and mixed with an equal volume of cold 16% polyethylene glycol (PEG) 6000 (Sigma Aldrich, Milan, Italy) in 1 M NaCl. The mixture was kept at 4 °C for 12 h and centrifuged at 10,000 x *g* for 20 min at 4 °C. The pellet containing EVs was resuspended in 8% PEG 6000 in 0.5 M NaCl and centrifuged in 2 mL tubes at 12,000 x *g*. Pellets were then resuspended in PBS (pH 7.4), obtaining a total of 1 mL of solution containing MVs from each isolation, and stored at -80 °C until their use in small aliquots to prevent multiple freeze-thaw cycles.

6.2.3 Nanoparticle Tracking Analysis (NTA)

The concentration and size of the isolated MVs were measured using Nanosight LM10 (Malvern System, Malvern, United Kingdom), equipped with a 405 nm laser. Each sample, once properly diluted (from 1:250 to 1:500 in water), was recorded for 60 s with a detection threshold set at maximum level. Temperature was monitored throughout the measurements. Videos were then subjected to NTA using NanoSight particle tracking software (version 3.2;

Malvern System, Malvern, United Kingdom) to determine nanoparticle concentrations and size distribution profiles.

6.2.4 Atomic Force Microscopy (ATM)

The topography corresponding to MVs was obtained by atomic force microscopy (AFM) analysis. Membrane vesicles of each strain were seeded and incubated on mica functionalized with polyornithine. After 30 min, a washing step was performed using 0.5 mL PBS and repeated for five times. Measurements have been performed in AC mode in liquid using an AC40TS cantilever (spring constant = 0.1 N/m, radius of curvature < 10 nm; Olympus corporation, Tokyo, Japan) on a MFP-3D Bio instrument (Asylum Research Technology, Oxford Instruments Company, Wiesbaden, Germany). Five fields of view of 5x5 μm^2 were acquired for each sample. The resulting images from the microscope were processed and analyzed using Gwyddion software (version 2.34; gwyddion.net).

6.2.5 Fatty acids composition

Fatty acids (FAs) analysis was performed on MVs and on their relative producer strain cells. LAB strains were grown at 37 °C for 24 h in MRS broth. The bacteria cells were harvested by centrifugation at 9,700 x g for 10 min, washed twice with phosphate-buffered saline PBS (pH 7.4) and resuspended in the same buffer. Absorbance at an optical density of wavelength of 600 nm (A_{600}) was adjusted to 0.10 ± 0.01 to standardize the number of bacteria (10^7 CFU/mL). The concentration of MVs was standardized to $5.8x10^{11}$ particles/mL. Fifty microliters for each MVs and bacterial solutions were used for the preparation of fatty acid methyl esters (FAMEs) that is reported in Buyer (2002). Analysis of FAMEs was carried out using a chromatograph Trace 1300 (Thermo Fisher Scientific, Milan, Italy) equipped with a split/splitless injector and a flame ionization detector. The separation was performed by capillary column HP-5MS UI, 30 m x 0.25 mm, 0.25 μm film thickness (Agilent Technologies, Santa Clara, California, USA). The operating conditions were: injection temperature, 280 °C; detector temperature, 300 °C; gradient: initial temperature, 140 °C; 5 °C min^{-1} from 140 °C to 300 °C. Helium was used as carrier gas at a flow rate of 1 mL min^{-1}. The injection volume was 1 μL and the split ratio was 1:10, and the samples were injected using an AL 1310 autosampler (Thermo Fisher Scientific, Milano, Italia). The identification of FAs was performed by comparing their retention time with respect to the standard Bacterial Acid Methyl Ester (BAME) Mix (Supelco, Sigma-Aldrich, Milan, Italy).

6.2.6 Protein quantification

Protein concentration was assessed using the Bicinchoninic Acid (BCA) protein assay kit (Novagen, Merck Millipore, Darmstadt, Germany).

6.2.7 RNA extraction and quantification

Total RNA from MVs was extracted with miRNeasy Tissue/Cells Advanced Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions and the concentration was determined using the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA).

6.2.8 Statistical analysis

Results from FAs analysis were analyzed with one-way analysis of variance (ANOVA) and Tukey's post hoc analysis at the ($p<0.05$) significance level. Similarities were underlined by the construction of Venn diagram. All the statistical analyses were performed using R software version 4.2.1.

6.3 Results and discussion

In this study, membrane vesicles produced by *L. paracasei* (LpMVs) and *L. rhamnosus* (LpMVs) were isolated and characterized. It has been demonstrated that lactic acid bacteria (LAB) MVs can produce a wide range of biological effects in the host recipient cells and may be partially responsible for the benefits provided by probiotic bacteria. Currently, there is a lack of knowledge on the cargo composition of Gram-positive bacteria and this constitutes a barrier to understanding the mechanisms of action underlying the probiotic effects.

6.3.1 Isolation of MVs

The isolation of MVs can be carried out using various methods based on different principles. Among them, differential ultracentrifugation (UC) is the most commonly used method for EVs isolation, but other methods can be also used in order to ameliorate the purity of the isolated MVs. At now, a gold standard method is not yet defined. Ultracentrifugation requires several centrifugation steps (> 100,000 x *g*) that allow the separation of small cellular debris and larger populations of vesicles (Mol et al., 2017). However, this is a laborious and time-

consuming method that requires expensive equipment. In this study, MVs were isolated using the methods proposed by Baüerl et al. (2020), which involves a polymer-based precipitation using PEG which is generally used for the isolation and concentration of viruses. Compared to UC, it provides higher yields of EVs efficiently and inexpensively without sacrificing their purity and reducing their biological activity (Rider et al., 2016; García-Romero et al., 2019; Ludwig et al., 2018).

6.3.2 Nanoparticle tracking analysis (NTA)

Suspensions containing isolated MVs were subjected to NTA, one of the most commonly used techniques for the rapid and high-throughput sizing and concentration determination of EVs. As shown in Figure 6.1, the size of LpMVs was homogeneous, as there was only one well-defined peak. The same can be stated also for LrMVs, although the peak was broader compared to that of LpMVs and the MVs size was higher. The size distribution mode was 30.25 ± 8.84 nm and 96.85 ± 5.58 nm for LpMVs and LrMVs, respectively. Analysis showed that the mean sizes were 70.3 ± 54.2 nm and 108.1 ± 46.7 nm for LpMVs and LrMVs, respectively. Particles that measure more than 200 nm in size can be the result of aggregation of single MVs (Yuana et al., 2015). Prior to analysis, MVs samples were observed under optical microscopy to exclude the possibility of bacterial contamination. Regarding other studies that measured MVs size using NanoSight instrument, LrMVs isolated in our study presented a similar size to those of *Lactobacillus crispatus* isolated by Wang et al. (2022). Ñahui Palomino et al. (2019) isolated MVs with mean diameters ranging from 133 nm to 141 nm from two strains of *L. crispatus* and one strain of *Lactobacillus gasseri*. Similar MVs size was recorded for MVs isolated from *Lactobacillus acidophilus*, *L. casei,* and *L. reuteri* (Dean et al., 2019). However, another *L. reuteri* strain presented a mean size of 260.5 nm, much higher compared to the other (Kim et al., 2022). It is worth noting that, in our study and several other studies, it has been performed a 0.2 μm filtration during EVs isolation procedure, to exclude the possibility of bacterial contamination. Therefore, EVs with a size higher than those of the filter pores have not been collected. Considering that the sizes of MVs produced by strains belonging to the same species are different, it might be think that this characteristic may depend on the strain. However, even using the same type of instrument, variability can be produced by different analysis settings including temperature, laser wavelength, duration of measurement, operator proficiency, ecc. (Gardiner et al., 2013).

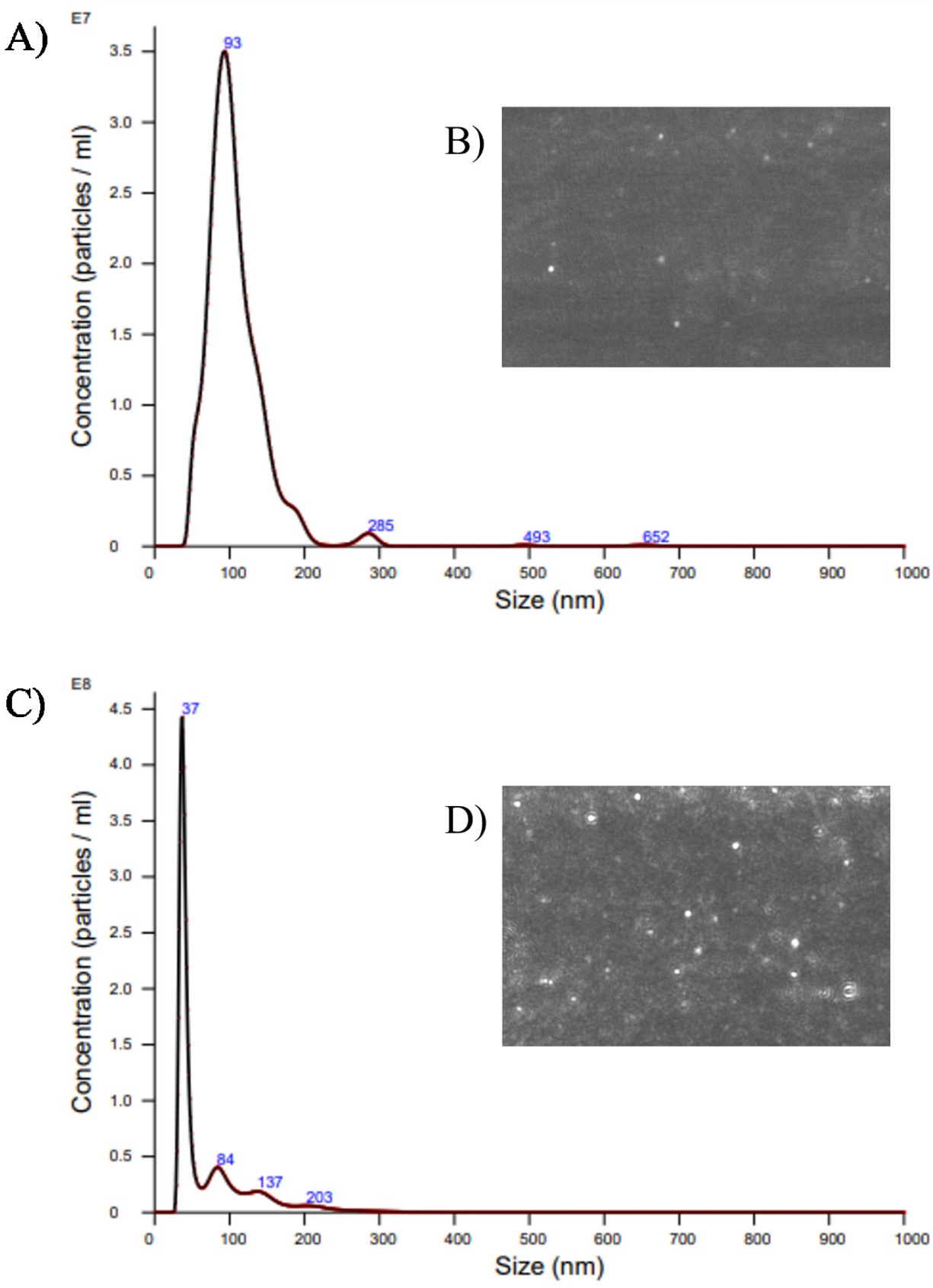

Figure 6.1 Representative nanoparticle tracking analysis (NTA) profiles showing size distribution of A) LrMVs and C) LpMVs, respectively. B) and D) are screenshots captured from the video recorded during NTA of LrMVs and LpMVs, respectively.

It can be concluded that the MVs produced by *L. paracasei* resulted higher in number but smaller in size compared to those produced by *L. rhamnosus*. Using NTA, was also measured the concentration of MVs in the samples that resulted to be $2.91x10^{12} \pm 1.7x10^{12}$ and $5.82x10^{11} \pm 1.63x10^{10}$ particles/mL for LpMVs and LrMVs, respectively. Currently, one single method that allows the determination of the size and concentration of the whole range of EVs does not exist and therefore a combination of different techniques is suggested (Erdbrügger & Lannigan, 2016).

6.3.3 Atomic force microscopy (ATM)

MVs isolated in this study were further imaged using AFM (Figure 6.2, 6.3). This microscopy technique, as well as electron microscopy (EM) provides a direct measurement of individual particle size and provides high-resolution visualization of EVs. Compared to EM techniques, AFM allows to analyze EVs in their native conditions, without complex sample preparation procedures (Gazze et al., 2021). Moreover, it can provide information regarding the biophysical (surface charge) and mechanical (stiffness and adhesion) properties of EVs. The immobilization conditions have a strong effect on the measurement of vesicle size. To better preserve the physical properties of EVs, especially their size, is better operating in liquid, the loss of water during the drying process required for the measurement in air may produce a shrinking of EVs size (Hardij et al., 2013; Parisse et al., 2017). Compared to those produced by *L. paracasei*, LrMVs were lower in number, according to the results obtained by NTA. The mean diameters were 110 ± 60 nm and 90 ± 50 nm for LrMVs and LpMVs, respectively.

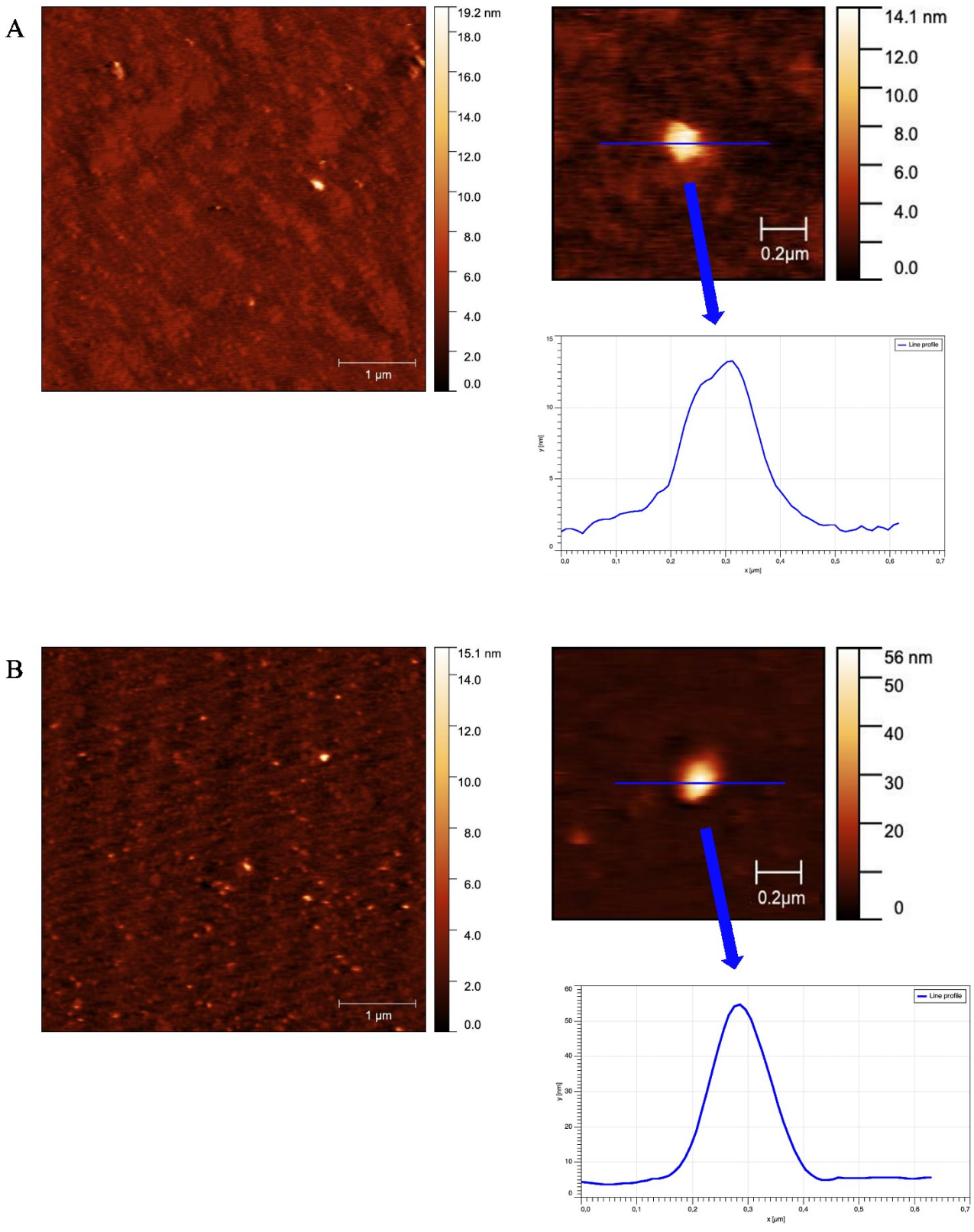

Figure 6.2 Representative images of atomic force microscopy (AFM) analysis of A) LrMVs and B) LpMVs, respectively.

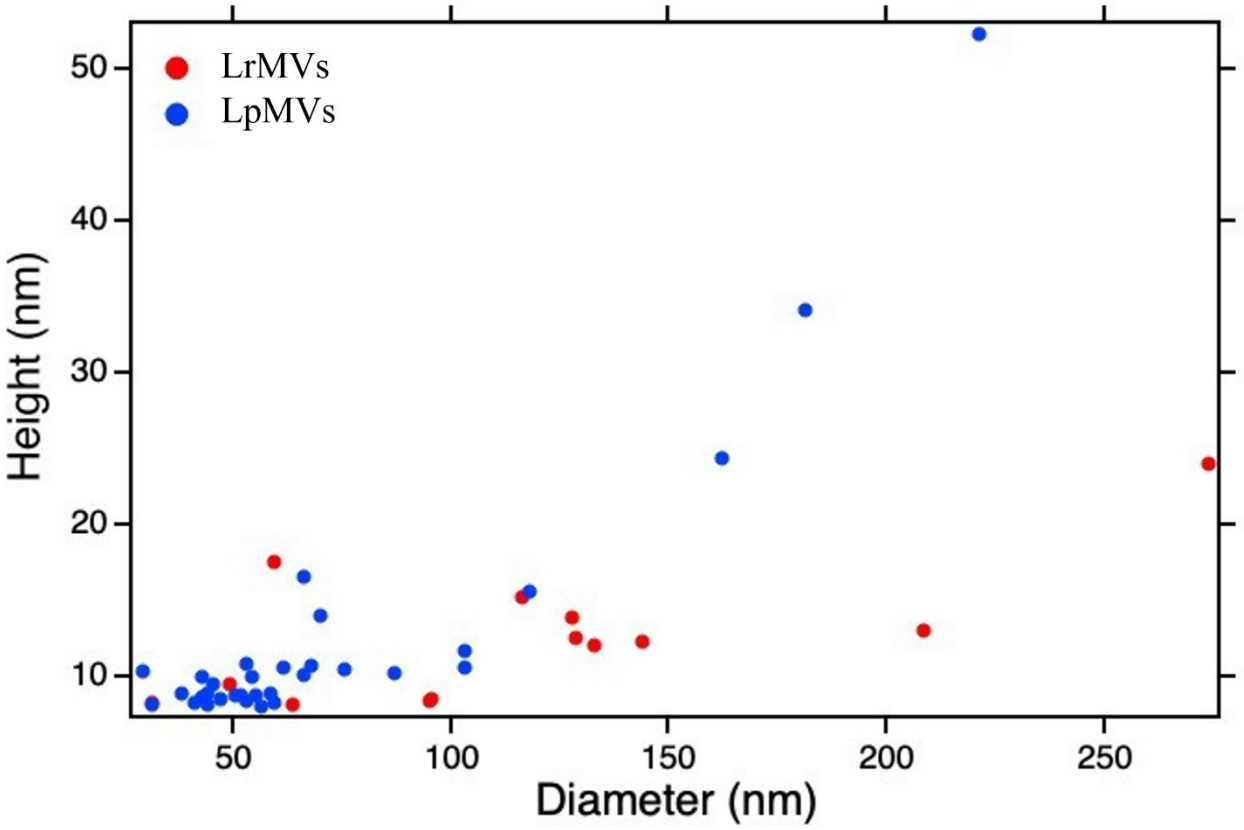

Figure 6.3 Scatter plot showing the distribution of LrMVs and LpMVs based on their diameter (nm) and height (nm) using AFM analysis.

6.3.4 Fatty acids analysis

Fatty acids analysis was performed on LpMVs and LrMVs, and, to be compared, on their respective producer strains (Figure 6.4). As previously mentioned, LAB MVs biogenesis is poorly studied and unclear, and probably, different possible mechanisms may be involved for different bacterial species. However, based on the studies carried out on other Gram-positive bacteria, it can be stated that the MVs membrane envelope derives from the cytoplasmic membrane of the bacterial cell (Briaud & Carroll, 2020). However, differences in the qualitative and quantitative lipid composition may be present, more or less marked, and it must be taken into account that FAs composition can be influenced by several factors including the growth medium, growth phase, salt concentration, temperature, and pH (Johnsson et al., 1995; Gautier et al., 2013). In this study, the most abundant FAs analyzed were palmitic acid (C16:0) and linoleic acid (C18:2 9-12c) both in LAB cells and MVs (Figure 6.4). Fatty acids of 16 and 18 carbons have been defined as the most abundant in LAB (Velly et al., 2015; Li et al., 2009; Johnsson et al., 1995).

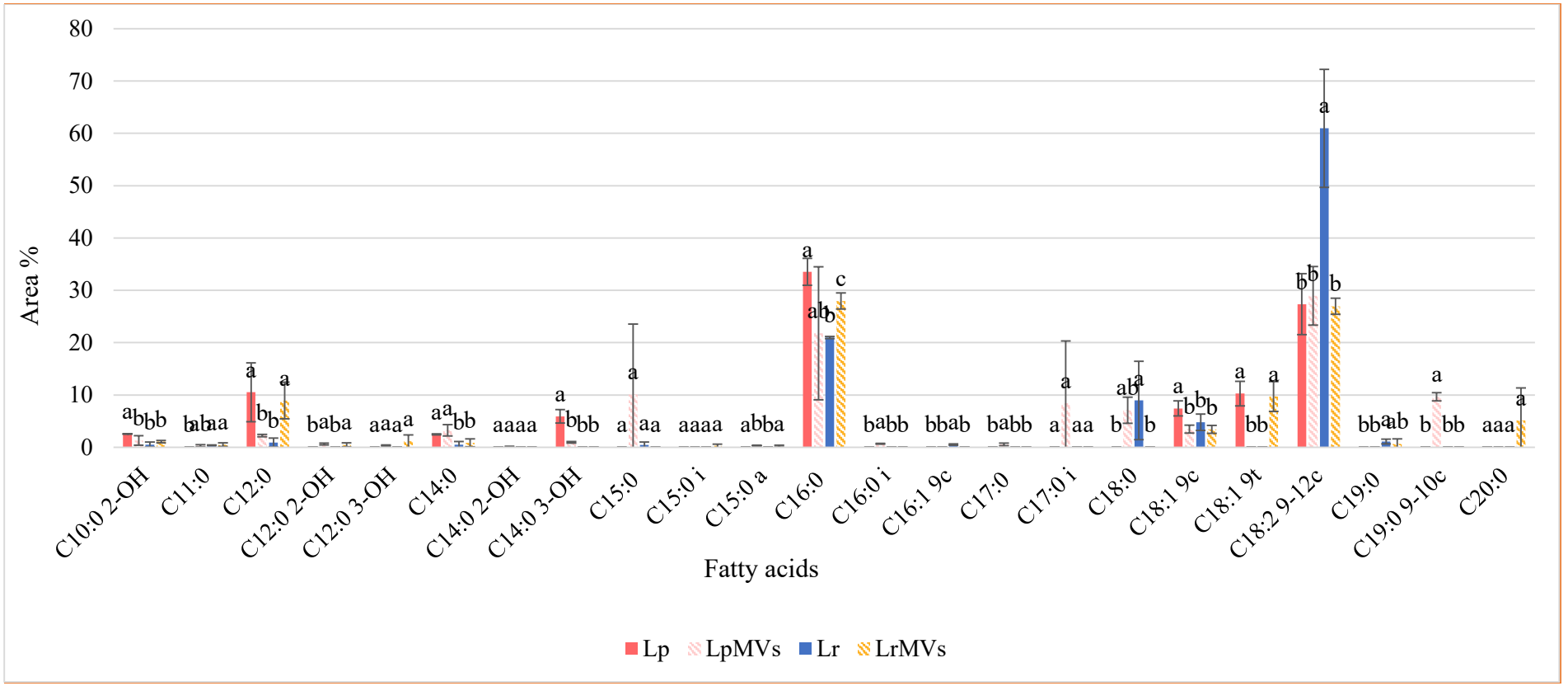

Figure 6.4 Composition of fatty acid profiles of *L. rhamnosus* (Lr) and *L. paracasei* (Lp) bacterial cells and their membrane vesicles (MVs). Bars represent the mean ± standard deviation (SD) of fatty acids expressed as relative abundance (%). Different letters (a-c) for the same fatty acids represent significant differences ($p<0.05$)

Similar composition between MVs and the parental cells, especially regarding the FAs present in high percentages, have been frequently observed also in other studies (Surve et al., 2016; Kurata et al., 2022, Rivera et al., 2010). This supports the assumption that the MVs membrane envelope originates from the cytoplasmic membrane of the producer bacterial cell. However, there were considerable differences in the minor FAs. It was suggested that differences in the fatty acid composition between MVs and the producer strain may be due to the promotion of the vesicle budding in specific lipid-enriched domains of the cytoplasmic membrane and lipids may be selectively enriched in MVs (Cao & Lin, 2021). It has been observed in MVs produced by *Listeria monocytogenes* and *Streptococcus pneumoniae* a specific enrichment in short-chain saturated FAs and unsaturated FAs which led to a high membrane fluidity that could facilitate the MVs release through the cell wall (Olaya-Abril et al., 2014; Coehlo et al., 2019; Cao & Lin, 2021). In our study this trend was not observed, indeed, although there was an increase of monounsaturated FAs in LrMVs compared to the producer strain, the amount of polyunsaturated FAs was considerably lower ($p<0.05$) (Figure 6.5).

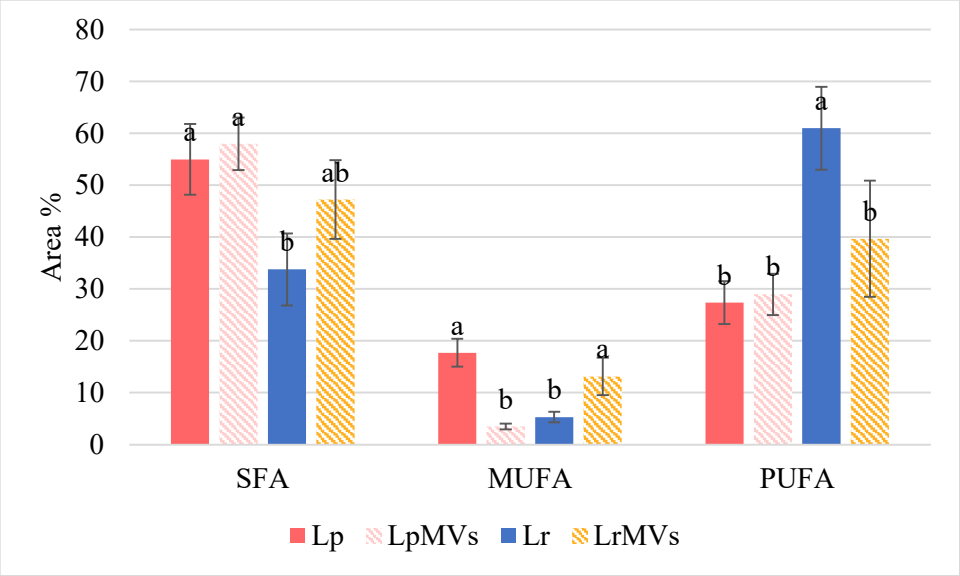

Figure 6.5 Composition in saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) of *L. rhamnosus* (Lr) and L.*paracasei* (Lp) bacterial cells and their membrane vesicles (MVs). Bars represent mean ± standard deviation (SD). Different letters (a,b) for each fatty acids group (SFA, MUFA, and PUFA) represent significant differences ($p<0.05$).

Resch et al. (2016) showed no differences between the amount of saturated and unsaturated FAs of *Streptococcus* and MVs produced. It must be taken into account that the analysis of FAs performed in this study cannot distinguish between MVs membrane FAs and internal

FAs. Therefore, the FAs detected may be part of the internal cargo of MVs, although most likely the great part of the FAs found both in MVs and strains belongs to the lipid bilayer membrane that envelopes the cells or the MVs. As can be seen in Figure 6.6, the number of FAs found in MVs is greater than that found in LAB cells and therefore MVs resulted to be more heterogeneous and diversified regarding FAs profile. Particularly, regarding *L. paracasei*, 8 and 18 different FAs were found in cells and MVs, respectively, regarding *L. rhamnosus* the differences were less pronounced since 11 and 14 FAs were found in cells and MVs, respectively (Figure 6.6). Almost all the FAs found in LAB cells were found also in the derived MVs except for oleic acid (C18:1 9t) in the case of *L. paracasei* and pentadecanoic acid (C15:0), palmitoleic acid (C16:1 9c), and stearic acid (C18:0) for *L. rhamnosus*, although there are significant differences regarding their abundance (%).

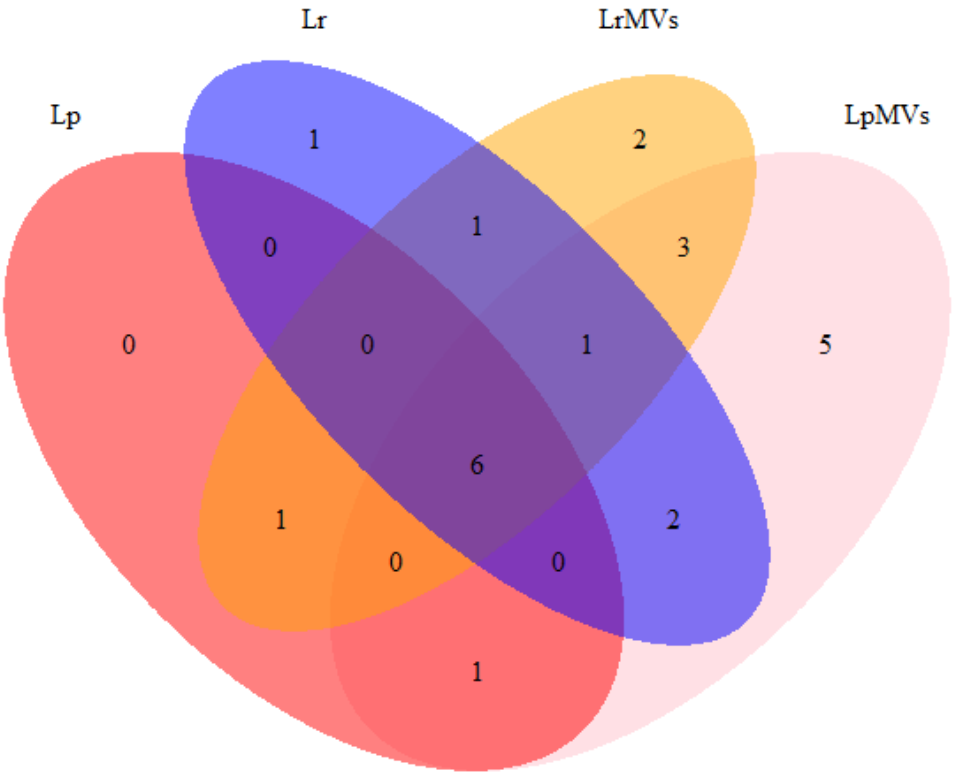

Figure 6.6 Venn diagram of the number of fatty acids detected in *L. rhamnosus* (Lr) and *L. paracasei* (Lp) bacterial cells and their membrane vesicles (MVs).

Regarding the FAs analysis, it should be underlined that the area of peaks relative to FAs of MVs samples were smaller than those coming from strain samples, probably due to the smaller size of MVs compared to the bacterial cells. Consequently, some peaks could be confused with the background noise. An optimization of the method in future studies is

required, especially regarding its sensitivity. Analysis of LAB MVs lipid composition has been performed less frequently compared to proteomic and transcriptomic analysis, probably because the beneficial effect of MVs is associated with the last two classes of constituents (Kim et al., 2020).

6.3.5 Protein quantification

During the biogenesis of MVs, the so-called vesiculogenesis, proteins and nucleic acids are packed into a lipid bilayer membrane and the biological functions of MVs are most probably related to these groups of macromolecules that represent the cargo of vesicles (Kurata et al., 2022). In this study, the protein concentration of the suspension containing MVs collected after each isolation was determined using BCA assay. *L. paracasei* MVs and *L. rhamnosus* had a protein concentration of 920.28 ± 55.18 and 185.89 ± 24.33 ng/µL, respectively. Proteomic analysis of membrane vesicles produced by lactobacilli was performed by several authors. Some proteins resulted to be exclusively present in MVs or bacteria whereas others were shared between MVs and the parental bacterial cell. Most of the proteins found in MVs are membrane transporter proteins, proteins involved in cell processes related to the cell surface, metabolic enzymes, proteases, and cell wall-associated hydrolases (Bajic et al., 2020; Domínguez Rubio et al., 2017; Dean et al., 2019; Lee et al., 2023). Moreover, the proteome analysis of MVs produced by *L. acidophilus* showed that they transport putative bacteriocins, demonstrating the potential antimicrobial activity that probiotics MVs may have on a complex microbial community such as the intestinal lumen (Dean et al., 2020).

6.3.6 RNA quantification

Another class of macromolecules that could be important for the biological function of EVs is RNA. Twenty microliters with an RNA concentration of 40.23 ± 0.87 and 2.95 ± 1.23 ng/µL was obtained by the extraction from 50 µL of the solution obtained after the isolation procedure of MVs of *L. paracasei* and *L. rhamnosus*, respectively. Regarding LAB MVs, small RNAs were sequenced from MVs produced by *L. plantarum* (Kurata et al., 2022; Yu et al., 2022). Yu et al. (2022) demonstrated the transfection of sRNA71, a microRNA present in *L. plantarum* MVs, in mammalian cells, and demonstrated its potential as a host cell apoptosis mediator. Studying the proteomic and transcriptomic will help to understand the potential of LAB MVs in bacteria-host communication and therefore will contribute to understanding the mechanisms of the beneficial effects of probiotics.

6.4 Conclusions

In this study, the ability to produce MVs was assessed for two type strains, *L. paracasei* and *L. rhamnosus*. Both strains were able to produce a considerable amount of MVs which were subsequently characterized. The potential biological function of MVs in modulating physiological conditions is triggered by their cargo. In the present study, proteins and RNA quantifications demonstrated that these two classes of components are present in MVs, and, in future studies, proteomic and transcriptomic analyses will be performed. Such analyses will contribute to understanding some of the mechanisms under bacteria and human host cells communication. This information is fundamental for the future development of new functional molecules for therapeutic applications.

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