Alma Mater Studiorum – Università di Bologna
in cotutela con Università di Granada

DOTTORATO DI RICERCA IN

Scienze e Tecnologie Agrarie, Ambientali e Alimentari

Ciclo XXXV

Settore Concorsuale: 07/I1 – MICROBIOLOGIA AGRARIA

Settore Scientifico Disciplinare: AGR/16 – MICROBIOLOGIA AGRARIA

Bio-preservation and bio-active compounds as sustainable strategies to improve quality and safety of fresh and fermented products

Presentata da: **Federica Barbieri**

Coordinatore Dottorato

Prof. Massimiliano Petracci

Supervisore

Prof. Fausto Gardini

Prof. Vito Verardo

Co-supervisore

Dott.ssa Giulia Tabanelli

Esame finale anno 2023

Summary

	Abstract	**1**
	Chapter 1: Introduction	**9**
1.1	**The Water-Energy-Food Nexus**	**11**
1.2	**Bio-protection: bio-protective cultures endowed with antimicrobial activity against the principal food-borne pathogens**	**12**
1.2.1	*Selection criteria of bio-protective cultures*	**13**
1.3	**Application of bio-protective cultures in food preservation: examples of biocontrol studies**	**18**
1.3.1	*Application in meat and meat products*	**19**
1.3.2	*Application in fish and seafood products*	**23**
1.3.3	*Application in other products categories*	**25**
1.4	**Starter cultures: technological advances for improving quality and safety of fermented products**	**26**
1.4.1	*Safety risks associated with fermented meat products*	**27**
1.4.2	*Screening of LAB strains regarding their safety aspects to select new candidate to be used as starter cultures*	**28**
1.4.2.1	Biogenic amines	**28**
1.4.2.2	Assessment of antibiotic-resistance profile	**30**
1.4.3	*Characterisation of LAB strains regarding their technological features*	**30**
1.4.3.1	Competitiveness of starter cultures	**31**
1.4.3.2	Acidification potential	**31**
1.4.3.3	Production of antimicrobial compounds	**32**
1.4.4	*Application of starter cultures in meat industry: two case studies*	**32**
1.4.5	*Application of starter cultures in fish products*	**33**
1.4.6	*Application of starter cultures in dairy products*	**35**
1.5	**Bio-preservation: antimicrobial effects of bio-active natural compounds to enhance food safety and extend shelf-life of food products**	**36**
1.5.1	*Application of bio-active compounds in meat products preservation*	**38**
1.5.2	*Application of bio-active compounds in fish products preservation*	**42**
1.5.3	*Application of bio-active compounds in dairy products preservation*	**44**
1.6	**Antioxidant and antimicrobial potential of unconventional matrices affected by drying and extraction methods**	**45**
1.6.1	*Recover of plant by-products to obtain added-value extracts to be applicated into food industry*	**45**
1.6.2	*Marine algae: an interesting source of bio-active compounds with a promising application as food ingredients*	**47**
1.6.3	*New extraction techniques for phenolic compounds recovery from plant by-products*	**49**
	Chapter 2: General aim	**51**
2.1	**General aim of PhD thesis**	**53**
	Chapter 3: Valorisation of spontaneously fermented sausages microbial	**59**

biodiversity
3.1 Mediterranean spontaneously fermented sausages: spotlight on microbiological and quality features to exploit their bacterial biodiversity 61
3.1.1 Introduction **61**
3.1.2 Materials and methods **62**
3.1.2.1 Spontaneously fermented sausages collection **62**
3.1.2.2 Physico-chemical analysis **62**
3.1.2.3 Microbiological characterisation **63**
3.1.2.4 DNA extraction, sequencing and bioinformatic analysis **64**
3.1.2.5 Biogenic amines determination **65**
3.1.2.6 Aroma profile analysis **66**
3.1.2.7 Statistical analysis **67**
3.1.3 Results and discussion **67**
3.1.3.1 Geographical origin and manufacturing processes **67**
3.1.3.2 Physico-chemical characterisation **69**
3.1.3.3 Microbiological analysis of the different spontaneously fermented sausages **71**
3.1.3.4 Metagenomic analysis **72**
3.1.3.5 Determination of biogenic amines content **78**
3.1.3.6 Fermented sausages aroma profile **80**
3.1.4 Conclusions **86**
3.2 Taxonomical identification and safety characterisation of *Lactobacillaceae* isolated from Mediterranean natural fermented sausages 89
3.2.1 Introduction **89**
3.2.2 Materials and methods **91**
3.2.2.1 LAB isolation and purification obtained from spontaneously fermented sausages samples **91**
3.2.2.2 DNA extraction and rep-PCR analysis to isolates identification **91**
3.2.2.3 Antibiotic-resistance profile determination and PCR-based screening of resistance genes **92**
3.2.2.4 Assessment of biogenic amines strains production **93**
3.2.3 Results and discussion **93**
3.2.3.1 Strain genotyping and identification of biotypes **93**
3.2.3.2 Safety assessment of isolated strains **97**
3.2.4 Conclusions **106**
3.3 Functional and technological characterisation of LAB strains isolated from Mediterranean spontaneously fermented sausages 109
3.3.1 Introduction **109**
3.3.2 Materials and methods **110**
3.3.2.1 LAB strains selected to perform antimicrobial activity against *E. coli* and *List. innocua* **110**
3.3.2.2 *In vitro* antimicrobial LAB strain activity to inhibit *E. coli* and *List. innocua* **111**
3.3.2.3 Antimicrobial LAB strains activity in meat model **111**
3.3.2.4 Gene-specific PCR analysis for the detection of bacteriocin encoding genes **113**

3.3.2.5 Selected strains employed for technological characterisation **113**
3.3.2.6 Study of LAB growth performances in presence of different salt concentrations and at different incubation temperature **114**
3.3.2.7 Aroma profile analysis of LAB strains in a real system **114**
3.3.2.8 Statistical analysis **115**
3.3.3 Results and discussion **115**
3.3.3.1 Antimicrobial activity of selected LAB strains against *E. coli* and *List. innocua*: *in vitro* tests **115**
3.3.3.2 Antimicrobial LAB strains activity in meat model (agar-salami medium) **116**
3.3.3.3 Detection of the presence of bacteriocins encoding genes **118**
3.3.3.4 Growth and acidification performances of selected LAB strains at different condition **119**
3.3.3.5 Aroma profile of selected LAB strains inoculate in a real food system **127**
3.3.4 Conclusions **132**
Chapter 4: Use of new LAB cultures in meat products: evaluation of their bio-protective activities and technological performances **135**
4.1 Application of autochthonous LAB as bio-protective cultures in fresh sausages **137**
4.1.1 Introduction **137**
4.1.2 Materials and methods **138**
4.1.2.1 LAB isolated form Spanish spontaneously fermented sausages and their identification **138**
4.1.2.2 Antimicrobial activity against food-borne pathogens **139**
4.1.2.3 Bacteriocins production and identification **139**
4.1.2.4 *In vitro* test to evaluate the growth of *List. monocytogenes* and *S. enterica* serovar Enteritidis in co-cultures with LAB strains **141**
4.1.2.5 Challenge test in fresh sausages **142**
4.1.3 Results and discussion **142**
4.1.3.1 Screening and selection of LAB strains isolated from Spanish spontaneously fermented sausages **142**
4.1.3.2 Effect of selected LAB strains against *List. monocytogenes* and *S. enterica* serovar Enteritidis growth kinetics **144**
4.1.3.3 Challenge test against *List. monocytogenes* and *S. enterica* serovar Enteritidis in fresh sausages **146**
4.1.4 Conclusions **150**
4.2 Use of two anti-listerial autochthonous starter strains to produce Spanish fermented sausages (salchichónes) **153**
4.2.1 Introduction **153**
4.2.2 Materials and methods **155**
4.2.2.1 LAB strains and growth conditions **155**
4.2.2.2 Salchichón production **155**
4.2.2.3 Physico-chemical and microbial analyses **155**
4.2.2.4 Evaluation of the aroma profile in the finished products **156**

4.2.2.5 Quantification of biogenic amines content **156**
4.2.2.6 Challenge test against *Listeria monocytogenes* **156**
4.2.2.7 Statistical analyses **157**
4.2.3 Results and discussion **157**
4.2.3.1 Physico-chemical and microbial characterisation of Spanish fermented sausages **157**
4.2.3.2 Detection of biogenic amines content **159**
4.2.3.3 Aroma profile of finished Spanish fermented products **160**
4.2.3.4 Challenge test against *Listeria monocytogenes* **162**
4.2.4 Conclusions **164**
Chapter 5: Study of the *in vitro* antimicrobial potential of bio-active compounds against spoilage microorganisms or food-borne pathogens **165**
5.1 Effects of *Rubus fruticosus* and *Juniperus oxycedrus* derivatives on culturability and viability of *Listeria monocytogenes* **167**
5.1.1 Introduction **167**
5.1.2 Materials and methods **168**
5.1.2.1 Plant collection and extraction of phenolic extracts (PE) and essential oils (EO) **168**
5.1.2.2 Characterisation of PE through HPLC analysis **169**
5.1.2.3 Characterisation of EO through GC–MS analysis **169**
5.1.2.4 Target microorganism strain and growth conditions **170**
5.1.2.5 Determination of minimum inhibiting concentration (MIC) **170**
5.1.2.6 Effect of plant derivatives on *Listeria monocytogenes* growth **170**
5.1.2.7 Flow cytometry analysis **171**
5.1.3 Results and discussion **172**
5.1.3.1 Characterisation of plant derivatives **172**
5.1.3.2 Effect of plant derivatives on *Listeria monocytogenes* growth kinetics **177**
5.1.3.3 Effect of plant derivatives on *Listeria monocytogenes* viability **181**
5.1.4 Conclusions **183**
5.2 Survival, growth and biogenic amine production of *Enterococcus faecium* FC12 in response to extracts and essential oils of *Rubus fruticosus* and *Juniperus oxycedrus* **185**
5.2.1 Introduction **185**
5.2.2 Materials and methods **187**
5.2.2.1 *Enterococcus faecium* FC12 **187**
5.2.2.2 Plant derivatives and minimum inhibiting concentration (MIC) determination **187**
5.2.2.3 Growth kinetics of *Ent. faecium* FC12 in the presence of tested plant derivatives **187**
5.2.2.4 Evaluation of biogenic amines production during samples incubation **187**
5.2.2.5 Flow cytometry analysis **188**
5.2.3 Results and discussion **188**
5.2.3.1 Evaluation of plant derivatives effects against *Ent. faecium* FC12 **188**
5.2.3.2 Determination of biogenic amines production **191**

5.2.3.3 Effect of plant derivatives on cell viability and culturability **193**
5.2.4 Conclusions **195**
Chapter 6: Increasing of bio-active compounds availability in vegetal matrices fermented by LAB strains **197**
6.1 Impact of LAB fermentation on phenolic compounds and antioxidant activity of avocado leaf extracts **199**
6.1.1 Introduction **199**
6.1.2 Materials and methods **200**
6.1.2.1 Avocado leaves collection and LAB strains used **200**
6.1.2.2 Avocado leaves fermentation **201**
6.1.2.3 Polar compound extraction **201**
6.1.2.4 Determination of Folin-Ciocalteu reacting substances **201**
6.1.2.5 Determination of antioxidant activity: DPPH and FRAP assays **201**
6.1.2.6 Determination of polar compounds by HPLC-ESI-TOF-MS **202**
6.1.2.7 Data processing **202**
6.1.3 Results and discussion **202**
6.1.3.1 Detection of LAB strains during avocado leaves fermentation **202**
6.1.3.2 Folin-Ciocalteu reacting substances content and antioxidant activity of the fermented avocado leaves **204**
6.1.3.3 Identification of polar compounds in fermented avocado leaves by HPLC-ESI-TOF-MS **206**
6.1.3.4 Quantification of phenolic compounds by HPLC-ESI-TOF-MS and their biotransformation during fermentation in avocado leaves **208**
6.1.4 Conclusions **213**
Chapter 7: General conclusions **215**
7.1 Conclusions **217**
Bibliography **221**

Abstract

Global projections reported that an increase in food demand of 35% will occur by 2030, mainly due to the population growth and economic development of emerging countries. In this perspective, a waste reduction in agro-food supply chains can be a fundamental strategy to guarantee environmental sustainability in food industry. This is possible through a valorisation of food residues, enhanced production yields and an extension of fresh food shelf-life. The increasing amount of food wastes is strongly related to the microbial spoilage, resulting in a loss of food quality and microbiological safety, especially in fresh or minimally processed products. The widespread consumers demand for natural and innovative foods directed the research towards the study of new "green" and environmentally friendly solutions, that need to be adapted to the specific characteristics of the different food products and processes. The development of sustainable approaches to assure safety, high quality and an increased shelf-life of both fresh and fermented products is a crucial aspect to pursue production efficiency and waste reduction. In particular, the attention has been focused on bio-preservation and bio-protection approaches, proposing, on the one hand, the use of natural antimicrobial compounds, and on the other, the application of microbial strains with antimicrobial properties toward pathogens and/or spoilage bacteria. In this latter case, nowadays, a limited number of commercial bio-protective and/or starter cultures are used to enhance product safety and quality. However, their application can cause product standardisation, with a loss in recognisability and peculiar features, linked to local productions. For this reason, an increased interest in traditional spontaneously fermented products as possible source of new autochthonous starter cultures or bio-protective strains for industrial purposes is emerging.

Given these considerations, this PhD project, that is part of the European project BioProMedFood, financed in the frame of PRIMA – Section 2 Programme, was focused on the study of sustainable approaches to improve quality and microbiological safety of fresh or fermented meat products. With this purpose, three main strategies have been evaluated. The first concerned the valorisation of spontaneously fermented sausages microbial biodiversity, through the isolation of autochthonous microbial strains and their use as bio-protective or functional starter cultures in meat products. In particular, the work started with the characterisation of 15 traditional fermented sausages, collected in Mediterranean countries, addressed to highlight their microbial biodiversity by using metagenomic analysis. Lactic acid bacteria (LAB) and coagulase-negative cocci were the most representative microorganisms in all samples, but significant differences were observed in relation to the product characteristics and origin. More than 900 LAB strains were isolated from these traditional products and approx. 150 biotypes, mainly belonging to the species *Latilactobacillus*

sakei, were detected. These results confirmed the high adaptation of this species to this ecological niche. These strains were studied regarding their antibiotic-resistances and amino biogenic potential and only safe strains (approx. 40) were characterised for their technological properties (growth performances at different salt concentrations and incubation temperatures) and their antimicrobial potential (inhibition of food-borne pathogens *in vitro* and the presence of genes related to bacteriocin production). The most promising strains were further studied for their anti-listerial activity in fresh sausages with promising results. In addition, they were used as starter cultures in the pilot plant production of fermented sausages. The results showed that the fermented sausages obtained with the selected strains were characterised by good aroma profiles and a reduced biogenic amine (BA) content. Moreover, the growth of *Listeria monocytogenes* during production and ripening, assessed with a challenge test, was strongly inhibited.

The second aim of the work was focused on the use of natural compounds with antimicrobial potential, extracted from plant derivatives of species characterising the Mediterranean maquis. In particular, phenolic extracts and essential oils of *Juniperus oxycedrus* needles and *Rubus fruticosus* leaves were tested *in vitro* against a strain of *List. monocytogenes* and *Enterococcus faecium*, also by using a cytofluorimetric protocol to better evidence the effect on viability and physiological state of target cells. In the latter strain, the inhibition of tyramine production was also assessed to evaluate the plant derivatives potential in reducing the accumulation of this toxic BA. Data collected underlined relevant differences in relation to species and type of plant derivatives, but they hint at important possibilities for applications in specific foods or processes.

The last topic of research of this thesis, carry out in collaboration with Department of Nutrition and Food Sciences (University of Granada), regarded the study of the effect of LAB fermentation on avocado leaves by-products. In particular, the attention was focused on the bio-availability of phenolic compounds in the plant extracts, caused by microbial metabolism. Even if the research work on this topic is still ongoing, the first data obtained are encouraging and an increase in antioxidant potential following LAB fermentation has been observed, even if in a strain-specific manner.

Concluding, the work presented in this PhD thesis permitted to highlight the great potential of traditional meat products to be a reservoir of microbial biodiversity and a source of isolation of new strains with industrial importance. Moreover, the antimicrobial potential of compounds obtained from under-exploited plant matrices was also investigated to assess their possibility to serve as "green" strategies in the increase of fresh food safety. These aspects demonstrated the potential use of innovative biotechnological strategies, to pursue production efficiency and promote the sustainability of the entire production system from a circular economy perspective.

Chapter 1:

Introduction

1.1 The Water-Energy-Food Nexus

Nowadays, water, energy and food resources are affected by a huge pressure due to the always increasing demand for their availability, caused by the actual global population growth, urbanisation, emerging economies development and climate change, that often lead to an environmental degradation and assets depletion. With respect to the 2015, global projections indicate that this issue can rise over 50% by 2050, stressing an already strong competition among these resources that are limited in many parts of the world (Ferroukhi et al., 2015; Zhang et al., 2018a). In response to this concern, the concept of the "water-energy-food Nexus" emerged to comprehensively assess the global resources systems with sustainable approaches, in order to avoid potential risks of uncertain future (Hoff, 2011). In fact, these three sectors are characterised by a complex interaction, such as in the case of biofuel. The enhanced development of bioenergy, promoted to mitigate climate change and replace fossil fuels, could be one of the potential causes of biodiversity loss and reduction of land dedicated to food crops (Meehan et al., 2010). Instead, regarding technological applications, for example, the water conserving irrigation and desalination techniques depend on an intensive energy consumption (Zhang et al., 2018a). For this reason, disregarding these interlinkages, the decision on how to manage one resource could impact not only on this latter, but also on the others, with unexpected and adverse consequences. The Food and Agriculture Organization (FAO) of the United Nations highlight that the function of the Nexus approach is to provide an overview of the current state and use of natural resources, in order to plan and implement their coordinated application to optimise synergies, efficiency and productivity (FAO, 2014). Despite the progress in water-energy-food Nexus, limitations of recent studies still exist, and this represents a challenge for the future research.

However, focusing on the food system, the population growth is leading to an increase in food consumption and consequent waste. This sector is characterised also by a significant volume of product waste during processing (one-third of the production), before reaching the consumer, because of the failure in the quality criteria (FAO, 2011; Ramírez et al., 2021). Food waste is related to the production country and local conditions. In low-income countries, this depends on managerial and technical limitations that affect post-harvest handling, storage and processing phases (FAO, 2021). In addition, also an overproduction can cause food waste (Beretta et al., 2013). Meanwhile, in the case of high- and middle-income countries, this aspect is linked to the distribution and consumption steps of the food supply chain.

In addition, this concern is strongly related to the microbial spoilage with a consequent loss in food quality and microbiological safety, in particular in fresh or minimally processed products, that results in human health risks, as well as high waste (FAO, 2015).

For these reasons, food waste represents one of the most challenging environmental, economic and safety issues of the 21st century and the knowledge of products degradation processes and storage mechanism are essential to achieve an effective use of resources (Tamasiga et al., 2022). Moreover, to overcome this problem, an improvement of these aspects and an extension of perishable food shelf-life could be necessary to obtain an efficient waste reduction and an optimisation of the production yields and technologies. In this perspective, Nexus approach can be considered the key to reach this purpose through strategies that promote these concepts in terms of sustainability (Hoff, 2011).

The widespread consumers scepticism regarding the addition of synthetic preservatives and the demand for natural and innovative foods, directed the research towards the study of new "green" and sustainable solutions to obtain healthy food products, maintaining their nutritional and sensorial properties and reducing microbial risks. In particular, the attention is focused on environmentally friendly techniques, such as bio-protection and bio-preservation. In the first case, bio-protective cultures, in particular lactic acid bacteria (LAB) strains, are used, while, in the second, bio-active natural compounds with strong antibacterial and antioxidant activities are employed, in order to reduce chemical and microbial degradation and prevent the proliferation and the presence of pathogenic microorganisms, with a consequent extension of shelf-life and microbial risks reduction.

1.2 Bio-protection: bio-protective cultures endowed with antimicrobial activity against the principal food-borne pathogens

In the past, the preservation of the most perishable foods (meat, fish, etc.) was ensured through ancient strategies, such as fermentation, in which products were submitted to spontaneously processes caused by the growth of indigenous microorganisms, present in the raw material. Moreover, salt and additives (*e.g.* nitrates and nitrites) were also added in considerably higher amounts than today, to prolong food storage for a long time (Toldrá and Hui, 2014). These strategies represented an effective hurdle against the principal spoilage microorganisms and food-borne pathogens.

On the other hand, nowadays, the consumers' demand for high quality, safe and minimal processed products, without the addition of chemical additives, directed the research and food industries towards alternative food processing and preservation technologies. In addition, the consumption of ready-to-eat (RTE) foods had a huge increase during the last decades (Bhattacharya et al., 2022). This type of products is characterised by an environment that could lead to potential outbreaks due to the associated microbial risk.

Despite the strict regulatory requirements and advances in food science and technology, the concern for the food-borne pathogens control in this type of products persists. For these reasons, alternative strategies to ensure an improvement of food safety and quality, with a consequent shelf-life extension, by using natural and environmentally friendly approaches are required. Among these techniques, bio-protective methods can represent an interesting solution to obtain this goal (Singh, 2018). These novel approaches consist in the use of selected safe microorganisms able to inhibit or prevent the growth of other undesirable, spoiling and pathogenic microorganisms by a direct competition or the production of specific antimicrobial metabolites (such as bacteriocins, organic acids, diacetyl, etc.).

An example of these biocontrol strategies is represented by the addition of bio-protective cultures able to prevent microbial and organoleptic deterioration of foods and prolong the shelf-life, without affecting product sensory properties (Oliveira et al., 2018). In this context, lactic acid bacteria (LAB) are the ideal candidates to be chosen as bio-protective cultures due to their key role and their long history of safe use in foods production. In fact, several strains belong to species that are recognised non-hazardous to human health with the GRAS (Generally Recognised As Safe) and/or QPS (Qualified Presumption of Safety) status from the US FDA and the EFSA, respectively (EFSA Biohaz, 2021). In addition, their potential as preservatives is explained by their capacity to produce a wide range of antimicrobial compounds (Gao et al., 2019).

This bio-protective strategy can be combined with other non-thermal technologies and mild treatments, to preserve the nutritional quality and properties of foods, fulfilling the increasing consumer demand for more natural and healthy products. On the other hand, it has been highlighted that both non-thermal technologies and LAB cultures application should be effectively optimised for each food production process, due to their huge differentiation (Rosario et al., 2020).

1.2.1 Selection criteria of bio-protective cultures

Bio-protective cultures are live microorganisms added deliberately into foods to microbiologically control the habitat, without affecting the organoleptic properties and sensory qualities of the products. For this reason, to be considered as bio-protective cultures, they necessarily must follow these criteria:

- Be classified as GRAS;
- Survive and remain active during product manufacturing, distribution and storage, as well as compete for nutrient and space;
- Do not produce any hazardous compounds to humans' health;
- Do not affect product intrinsic characteristics (sensory properties, flavour and texture);

- Be able to inhibit the growth of spoilage and/or pathogenic microorganisms by natural antimicrobial compounds production (Ben Said et al., 2019).

As regards the last point, numerous LAB are able to produce bacteriocins, low molecular weight peptides (20-60 amino acids) ribosomally synthesised, which play an important role in food preservation due to their bactericidal or bacteriostatic broad- or narrow-spectrum activity, at precise concentrations, against target microorganisms (Yilmaz, et al. 2022).

Bacteriocins are characterised by a huge diversity in term of primary structure, composition, physico-chemical properties and way of action and release. Indeed, over years, researchers regrouped these peptides in different classes, depending on their size, molecular composition and structure or modification (Simons et al., 2020). Firstly, these compounds were classified regarding the type of the producing microorganisms: Gram-positive or Gram-negative bacteria (Figure 1.1).

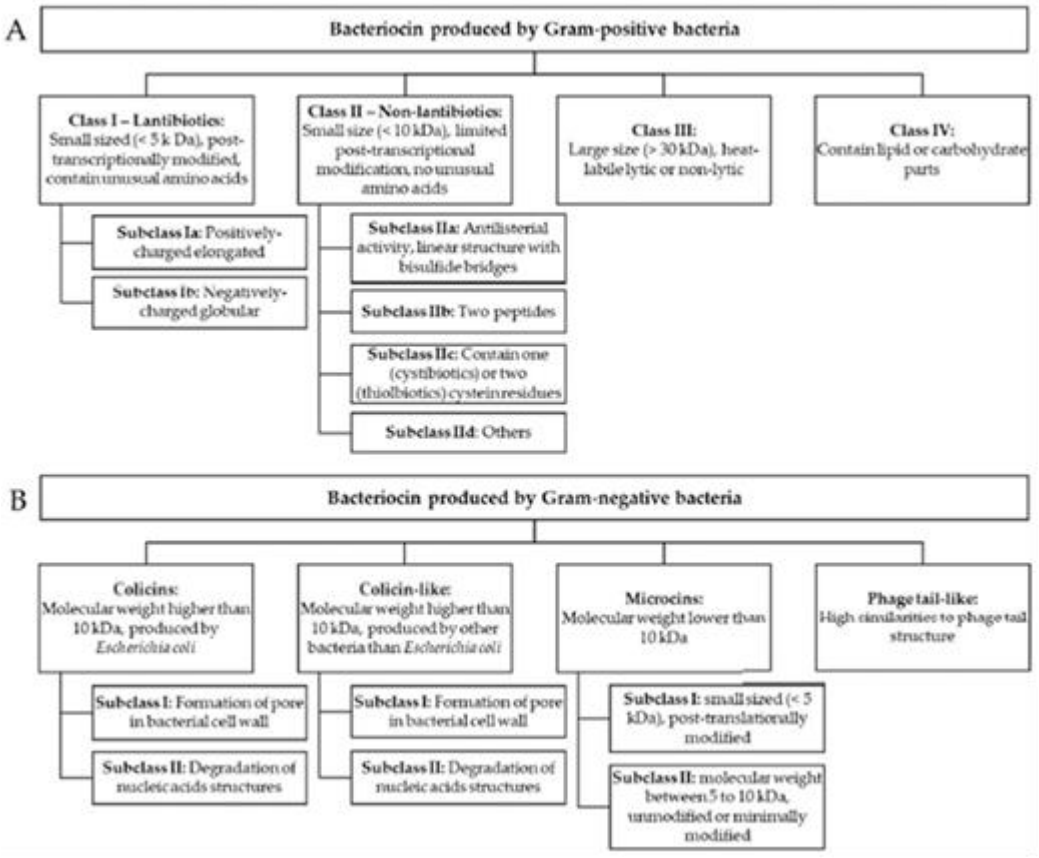


Figure 1.1: Classification of bacteriocins produced by Gram-positive (A) and Gram-negative (B) bacteria (Simons et al., 2020).

Focusing on the classification of bacteriocins produced by Gram-positive bacteria, they were commonly classified in four different groups (Zacharof and Lovitt, 2012). However, according to the classification outlined by Cotter et al. (2013), these compounds were mainly categorised into two classes, in which the smallest peptides were characterised by those with molecular mass

< 5kDa and the presence of post-translationally modification (class I) or unmodified bacteriocins of 6-10 kDa (class II). Based on this, Soltani et al. (2021) proposed an updated of this latter classification (Table 1.1). The modifications make generally class I bacteriocins more stable to high temperatures, extreme pH or proteolytic enzymes than those of class II. Moreover, class II is further subdivided into three subclasses: pediocin-like bacteriocins, non-pediocin-like bacteriocins and two-peptide bacteriocins.

Table 1.1: Classification of bacteriocins in two classes of post translationally modified and unmodified peptides (adapted from Soltani et al., 2021).

Class I	Characteristics	Examples of bacteriocins (producer strain)
Lantibiotics	Contain dehydroamino acids (lanthionine and 3-methyl-lanthionine) resulting from dehydration of serine and threonine residues, which form thioether β-carbon linkages with cysteines. Some require two lantibiotic peptides to be functional (two-peptide lantibiotics).	Nisin (*Lactococcus lactis*) Mutacin 1140 (*Streptococcus mutans*) Lacticin 3147 (LtnA1/LtnA2) (*Lc. lactis* DPC3147)
Linaridins	Linear peptides that contain dehydroamino acids, allo-isoleucine, N-terminal N,N-dimethyl-alanine and C-terminal 2-aminovinyl-D-cysteine.	Cypemycin (*Streptomyces* sp. OH-4156)
Thiopeptides	Macrocyclic peptides that contain a characteristic six-membered nitrogen-containing ring, oxazole/thiazol(in)e rings and/or dehydroamino acids	Thiostrepton (*Streptomyces azureus*)
Circular peptides	N-to-C cyclised unmodified single peptides	Enterocin AS-48 (*Enterococcus faecalis* S-48) Garvicin (*Lactococcus garvieae*) Gassericin A (*Lactobacillus gasseri*)
Bottromycins	Macrocyclic peptides with a linear tail that contain an amidine moiety, methylated amino acids and a C-terminal decarboxylated thiazole	Bottromycin A2 (*Streptomyces bottropensis*)
Class II	**Characteristics**	**Examples of bacteriocins (producer strain)**
Pediocin-like single peptides	Contain the YGNGV consensus sequence and with anti-listerial activity	Pediocin PA-1 (*Pediococcus acidilactici*) Enterocin CRL35 (*Enterococcus mundtii*) Sakacin A and P (*Latilactobacillus sakei*) Curvacin (*Latilactobacillus curvatus*) Leucocin A (*Leuconostoc gelidum UAL187*) Carnobacteriocin BM1 (*Carnobacterium piscicola* LV17B)
Unmodified single peptides	Unmodified linear single peptides that do not contain the YGNGV consensus sequence	Lactococcin A (*Streptococcus cremoris*)
Two-peptides	Require two or more unmodified peptides to be functional	Plantaricin F (*Lactiplantibacillus plantarum*) Lactacin F (*Lactobacillus johnsonii*)

Several bacteriocins remain stable in low pH condition, high temperature and at different salt concentration (Yang et al., 2018). The advantages linked to their addition into food products, that does not affect organoleptic properties, concern the possibility to extend shelf-life of foods by reducing the microbial risk related to the presence of sensitive food-borne pathogens through the food chain and enhancing the bio-protection during storage. These peptides can work with two different mechanisms of action: in particular, they can inhibit the cell wall synthesis due to the

interaction with lipid or cause pore formation into microbial membrane (Egan et al., 2016). Moreover, the use of bacteriocins could allow the application of less severe treatments during processes, that result in a better preservation of foods nutrients and sensory properties, without compromising their safety (Soltani et al., 2021). Therefore, the economic losses due to food spoilage, recalls or outbreaks could be reduced.

Despite the huge potential of the bacteriocins application in food industry has been extensively reviewed (Ben Said et al., 2019), only nisin, produced by *Lactococcus lactis*, and pediocin PA-1, produced by *Pediococcus acidilactici*, are currently approved by regulatory agencies as a food preservative (Oliveira et al., 2018; Soltani et al., 2021). In particular, nisin is widely used in dairy industries (12.5 mg/kg) to control clostridia and *Listeria* contaminations (Favaro et al., 2015; Reis et al., 2012), but this and other bacteriocins have been shown to inhibit spoilage or pathogen microorganisms in different food matrices, including meat and meat products, seafoods and fish products, beverages, vegetables and cereals (Ben Said et al., 2019).

Bacteriocins can be added into food products as semi-purified peptides, generally containing other antimicrobial compounds (such as organic acids). The effectiveness of their activity is strongly influenced by the food matrix characteristics and ingredients (proteins, lipids, pH, etc.). In fact, a limited and non-homogeneous distribution of these compounds or an inactivation by proteolytic enzymes can significantly affect their antimicrobial activity. To overcome these issues, other potential strategies are developed. In particular, encapsulation technology could provide stability and facilitate controlled release of bacteriocins in food matrices (Chandrakasan et al., 2019). Bacteriocins can also be incorporated into food packaging films to inhibit the spoilage or growth of pathogenic microorganisms during the storage period. Through this innovative strategy bacteriocins improve food safety without interacting with its ingredients, preventing the inactivation risk (Aymerich et al., 2022; Damania et al., 2016). Focusing on meat industry, bacteriocins have represented an attractive alternative to improved safety of fermented meat sausages and were extensively explored. Marcos et al. (2013) studied the antimicrobial effect of packaging film containing nisin against *Listeria monocytogenes* survival on sliced fermented sausages. The results showed a strong reduction (4.57 log cfu/g) of *List. monocytogenes* when bacteriocin was incorporated into packaging. Other studies were performed to characterise and test bacteriocins, alone or in combination with other hurdles, to control undesirable microorganisms in fermented sausages (Table 1.2). Most of these researches were carried out to control *List. monocytogenes*, showing a reduction of its cell number below the detection level.

Table 1.2: List of several study regarding biocontrol strategies in Mediterranean-style fermented sausages (adapted from Oliveira et al., 2018).

Bacteriocin (producing strain)	Product	Method	Target microorganism	Note	References
Enterocins 416 K1 (*Enterococcus casseliflavus* IM 416 K1)	Italian dry fermented sausage (*ciacciatore*)	Addition of bacteriocin as ingredient (10 AU/g) or bacteriocin-producing culture (5 log cfu/g)	*List. monocytogenes*	2.09 log cfu/g decrease for *List. monocytogenes*; no inhibition was observed when enterocin was incorporated alone.	Sabia et al., 2003
Enterocins A and B (*Enterococcus faecium* CTC492)	Spanish dry fermented sausage (*fuet*)	Addition of bacteriocin as ingredient (2000 AU/g); Combination with HHP (400 MPa)	*List. monocytogenes* *Salmonella enterica* *Staphylococcus aureus*	The HHP treatment *S. enterica* (< 1 log cfu/g), but only the combination of enterocins and HHP reduce *List. monocytogenes* to this level; *Staph. aureus* was not inhibited.	Jofré et al., 2009
Enterocins AS-48 (*Ent. faecalis* S-48)	Spanish dry fermented sausage (*fuet*)	Addition of bacteriocin as ingredient (148 AU/g); Combination with HHP (400 MPa)	*List. monocytogenes* *S. enterica* *Staph. Aureus*	5.5 log cfu/g decrease for *List. monocytogenes* and 1.79 log cfu/g for *S. enterica* at the end of ripening; no effect of enterocin or HHP was observed for *Staph. aureus.*	Ananou et al., 2010
Nisin (*Lc. lactis*)	Turkish dry-fermented sausage (*sucuk*)	Addition of bacteriocin as ingredient (5, 10, 25, 50 and 100 µg/g)	*List. monocytogenes*	No surviving cells were detected with 50 and 100 µg/g of nisin concentration at 20 and 25 days, respectively.	Hampikyan and Ugur, 2007
Bacteriocin MBSa2 (*Lat. curvatus*)	Italian type salami	Addition of bacteriocin as ingredient (200 AU/g)	*List. monocytogenes*	2 and 1.5 log cfu/g reduction of *List. monocytogenes* at 10 and 20 days, respectively.	Barbosa et al., 2015
Pediocin bacHA-6111-2 (*P. acidilactici* bacHA-6111-2)	Portuguese semi-dry fermented sausage (*alheira*)	Addition of bacteriocin as ingredient (320 AU/g) or bacteriocin-producing culture (8 log cfu/g); Combination with HHP (300 MPa)	*Listeria innocua*	Synergic effect between both incorporation method with HHP.	Castro et al., 2018

Furthermore, also in the case of fish products these studies were performed. For example, divergicin M35, the bacteriocin produced by *Carnobacterium divergens* M35, was able to inhibits growth of *List. monocytogenes* and *Staph. aureus* on cold-smoked salmon (Tahiri et al., 2009)

Although bacteriocins represent an attractive solution to improve safety aspects in food products, in European Union the use of purified or semi-purified bacteriocins must be labelled as additives (*e.g.* nisin E number E234) and this could be controversial for the consumers request (Oliveira et al., 2018). Moreover, as the products manufacture can affect the performances of bacteriocins, it is necessary to deeply know how these antimicrobial compounds could be inactivated and develop new enforcement strategies.

In this respect, a feasible alternative could be represented by the application of bacteriocin-producing LAB strains, directly inoculated into food products as a bio-protective cultures (Ben Said et al., 2019). In addition, other antimicrobial compounds could be produced due to the growth of these microorganisms into foods. In particular, organic acids (lactic and acetic acid) can be generated by carbohydrates fermentation in different amount, depending on the species and growth conditions. Their accumulation in the extracellular environment causes a decrease in pH that could

lead to an acidification stress with consequent antimicrobial effect: acids can diffuse passively through the cell membrane and lower the intracellular pH. This can cause a reduction in the activity of acid-sensitive enzymes and damages in proteins and DNA. Finally, LAB species can produce some metabolites, such as volatile compounds, that provide not only an impact on the aroma profile of the product but also show an inhibitory effect against both Gram-positive and Gram-negative bacteria, including the principal food-borne pathogens. An example of this is represented by diacetyl (2,3-butanedione), commonly associated with dairy products (Ben Said et al., 2019).

Several works have focused the attention on the direct use of LAB as a bio-protective strains against food-borne pathogens, including *List. monocytogenes*, *Salmonella* spp., *Escherichia coli* O157:H7 and *Staph. aureus*, in different food products, underlining the promising aspect of this application.

1.3 Application of bio-protective cultures in food preservation: examples of biocontrol studies

Trends in food consumption and technology and the consequent intensification of food production impact on microbial safety, resulting in a loss of quality features of products. This issue, depending also on the persistence of spoilage microorganisms and food-borne pathogens, led to discarding of 30-40% of food directed to consumer use and significant economic losses. The serious consequences caused by food-borne pathogens and their associated outbreaks constitute a critical point not only in the food industry, but also for the global public health (Zapaśnik et al., 2022). Despite technological progress, the ever more challenging reduction of the impact of these undesirable microorganisms drove the research towards innovative and safe methods for food preservation, including the application of bio-protective LAB cultures (Daliri et al., 2020). Many studies demonstrated the inhibiting effect of LAB against the growth of the most concern pathogens, such as *List. monocytogenes*, *Salmonella* spp. and *E. coli*, without changing in sensory characteristics of the products (Castellano et al., 2017).

During their growth, LAB strains could produce different metabolites characterised by an antimicrobial activity. They could induce the membrane destabilisation, the inhibition of the synthesis of cell wall enzymes, the interference of proton gradients and the increase in oxidative stress (Pradhan et al., 2020). Primarily, food-borne pathogens are efficiently inhibited due to pH reduction, that depends on the production of organic acid by LAB cultures, in particular lactic or acetic acid (Stanojevic-Nikolic et al., 2015). In addition, bacteriocins produced by LAB can also contribute to the inhibition of these adverse microorganisms. Nevertheless, it is necessary carry out further studies regarding LAB effectiveness linked to environmental conditions of specific food

products and better assess their application in the food chain. Therefore, a huge number of research works and reviews about the application of bio-protective cultures into several categories of food products are performed in order to enhance their safety aspect and to prolong shelf-life.

1.3.1 Application in meat and meat products

Meat and meat products represent an important nutritional source for human diet (Das et al., 2020). Nevertheless, these products are perishable and highly susceptible to microbial contamination and physico-chemical deterioration, such as undesirable changes in the quality, and alters colour, texture and flavour (Biswas et al., 2021). Meat fermentation is considered a hurdle approach that can guarantee food safety, ensured by the presence of multiple factors and specific chemical conditions, such as pH, water activity (a_w), sodium chloride nitrate and nitrite, that can act in limiting microbial growth (Leroy et al., 2013; Siddi et al., 2022). Otherwise, despite the rigorous hygienic and technological standards, the final products are still not exempted from microbial hazards during process and storage (EFSA, 2015). The consumption of meat products, also RTE, contaminated with pathogenic microorganisms are often linked to several food-borne outbreaks (Abebe et al., 2020; Neri et al., 2019). *List. monocytogenes* is one of the most relevant pathogens that can occur in RTE foods, due to the drastic consequence of listeriosis disease in immunocompromised individuals and pregnant women (CDC, 2017). For example, in 2019 a large outbreak of listeriosis linked to the consumption of a chilled roasted pork meat product is reported in Spain with a total of 217 reported cases, in which 3 deaths and 6 women that had miscarriages linked to this outbreak are noted (CCAES, 2019). According to the report published in 2021 by EFSA, *List. monocytogenes* was considered the fifth most commonly reported zoonosis agent, with 1876 confirmed human cases of listeriosis and 16 outbreaks, referring to 2020 (EFSA, 2021). In addition, spoilage bacteria can also compromise meat products shelf-life.

With the high rate of these disease and the meat predisposition to the incidence of undesirable microorganisms, many studies are performed to ensure food safety and increase products shelf-life by using LAB cultures, demonstrating their effectiveness as a bio-protective strategies (Bhattacharya et al., 2022; Bintsis, 2018). Several works are focused on their application in meat-based products against the principal food-borne pathogens or spoilage microorganisms, such as *List. monocytogenes*, *Salmonella* spp., *E. coli*, *Clostridium* spp., *Campylobacter jejuni* and *Brochothrix thermosphacta*. In particular, most of these are focused on the control of the presence of *List. monocytogenes*, implementing some technological innovations in order to ensure that its level of contamination must not exceed 100 cfu/g up to the end of the shelf-life, provided that these products do not support the growth of the pathogen, as established by the EU legislation with the

Regulation EC No. 2073/2005 (European Commission, 2005). According to the EU regulation, dry-fermented sausages with pH $\leq$ 4.4 or $a_w \leq 0.92$ and pH $\leq$ 5 or $a_w \leq 0.94$ are automatically considered to belong to food category 1.3 of RTE food unable to support the growth of *List. monocytogenes*. On the contrary, more restrictive criteria are required by the US regulation, in which this pathogen must not be detected (zero tolerance) in RTE meat products released in the market (FSIS, 2012).

Among LAB group, the application of species belonging to *Enterococcus* genus in food systems could be interest since they can produce bacteriocins (enterocins) with antagonist activity against a wide range of undesirable microorganisms. Despite this, due to their association with some food-borne outbreaks, they were removed from FAO's GRAS list, even if this unsafe behaviour is strain dependent and not linked to the whole genus (Khan et al., 2010). In fact, enterococci have a long history of safe use in food industry thanks to their role to determine required sensory characteristics, as well as to produce bacteriocins (Giraffa, 2003; Todorov et al., 2022). Considering their previously reported safety status, some researches have been focused on the application of enterococci, that did not present virulence factors, in food bio-protective approaches. In particular, the effectiveness for the inhibition of *List. monocytogenes* by different strains of *Enterococcus mundtii*, isolated from the Patagonian environment, was studied. Orihuel et al. (2018) focused their work on the study of *Ent. mundtii* CRL35, producer of enterocin CRL35 with strong anti-listerial activity, in a meat model. The results underlined its high antimicrobial performance against *List. monocytogenes* in a cured meat environment. Other *Ent. mundtii* strains were tested again to counter *List. monocytogenes* during refrigerated storage in ground beef. They significantly reduced the growth of this target microorganism over the days (Gomez et al., 2021). Both these studies indicated that these strains could be used as bio-protective culture in meat industry, ensuring hygiene and quality of the final product.

However, the main species associated with meat products, in particular fermented ones, are represented by *Lat. sakei*, *Lat. curvatus* and *Ltp. plantarum*, that are dominant during fermentation and ripening process (Cocolin et al., 2011). Regarding *Lat. sakei* species, in the past Katikou et al. (2005) demonstrated that the strain L115 produced bacteriocin-like substances with inhibitory effect against *List. monocytogenes* growth, that also significantly reduced the levels of spoilage microorganisms in beef slices during refrigerated storage. Recently, the potential activity of the same LAB strain was evaluated *in vitro* against a five-strain cocktail of *List. monocytogenes*. The results showed that the growth rate of the target pathogen significantly decreased in the presence of *Lat. sakei* L115 (Costa et al., 2020). Otherwise, the impact of this behaviour should be assessed into

food matrices. On this matter, Oliveira et al. (2018) in their review summarised several previous works about the application of *Lat. sakei* in fermented meat products (Table 1.3).

Table 1.3: List of strains used as bio-protective cultures in Mediterranean-style fermented sausages (adapted from Oliveira et al., 2018).

Bio-protective culture	Product	Mechanism	Target microorganism	Note	References
Lat. sakei (2 autochthonous strains)	Portuguese dry-fermented sausage (*chouriço de vinho*)	Antagonistic activity against pathogens	*List. monocytogenes*	Reduction of the pathogens during the processing, leading to counts below the detection limit	Linares et al., 2013
Lat. sakei ST153 *Ltp. plantarum* ST202	Portuguese semidry fermented sausage (*alheira*)	Bacteriocinogenic strains against *List. monocytogenes*	*List. monocytogenes*	2 log cycles reduction on listerial population when *Lat. sakei* ST153 was used	Vaz-Velho et al., 2013
Lat. sakei ST153 and BLC35	Dry-fermented Portuguese sausage (*chouriço*)	Bacteriocinogenic strains against *List. monocytogenes*	*List. monocytogenes*	Reduction of *List. monocytogenes* to values lower than 100 cfu/g after application of LAB	Jácome et al., 2014
Lat. sakei CTC494	Iberian dry fermented sausage (*chorizo*)	Bacteriocinogenic strains: *Lat. sakei* - sakacin K	*List. monocytogenes*	5.4 log reduction on listerial counts for *Lat. sakei* after 14 days	Ortiz et al., 2014
Lc. lactis ssp., *Lacticaseibacillus casei*,	Italian dry fermented Sausage (*Salame nostrano*)	Strains chosen on the basis of previous in vitro tests with selected pathogens	*Cl. botulinum*, *Enterobacteriaceae*, *Listeria* spp., *Pseudomonas* spp., *E. coli*, *Salmonella* spp., *Staph. aureus*	The growth of *Staph. aureus*, *Pseudomonas* spp., *Listeria* spp. and *Enterobacteriaceae* was inhibited in salami with the starter cultures	Cenci-Goga et al., 2016

More recently, the behaviour of *List. monocytogenes* in two types of chicken-based dry-fermented sausages was assessed during the fermentation and ripening process by Austrich-Comas et al. (2022), through the addition of the bio-protective culture *Lat. sakei* CTC494. This strain is sakacin K producer, a bacteriocin characterised by an anti-listerial effect that has demonstrated to reduce the target microorganisms up to 2 log cycle *in vitro* (Ortiz et al., 2014). However, the effectiveness of a bio-protective culture depends on the production process conditions and on the food matrix properties. For these reasons, it is necessary to assess its performances case-by-case, with a final aim to guide food industries through the research of the more efficient application of these strategies (Barcenilla et al., 2022a).

In the study of Austrich-Comas et al. (2022), chicken meat was inoculated with a cocktail of three *List. monocytogenes* strains and stuffed into small (*snack*-type) or medium (*fuet*-type) casings. *Lat. sakei* CTC494 was able to prevent the pathogens growth (that in the control samples was more than 3 log cfu7g) in the *snack*-type sausages and enhanced their inactivation in *fuet*-type sausages (reduction of 1.55 log cfu/g). These results could be related to the earlier acidification and

consequent decrease of pH linked to the higher lactic acid production, but also to the production of the anti-listerial bacteriocin sakacin k.

In another study, Serra-Castelló et al. (2022) evaluated the effect of the same bio-protective culture (*Lat. sakei* CTC494) against *List. monocytogenes* in another category of meat product, a vacuum-packaged sliced cooked ham, to ensure safety of this product during shelf-life. The results showed a complete inhibition of the pathogen growth especially in the condition characterised by the highest concentration of LAB inoculum (5 log cfu/g) and the lower storage temperature tested.

Other *in vitro* studies were performed by using *Lat curvatus* 54M16, isolated from traditional fermented sausages of Campania region (Casaburi et al., 2016). This strain can produce more than one bacteriocin, carrying the genes for sakacin X, T and P as demonstrated by PCR analysis performed in this work. All the *in vitro* conditions tested for the production of bacteriocins (temperature, pH and NaCl) indicate that *Lat. curvatus* 54M16 strain was able to grow and produce these antimicrobial compounds, to which target microorganisms *List. monocytogenes*, *Bacillus cereus* and *B. thermosphacta* were sensitive. These results encourage further *in vivo* studies that will carry out with the aim to assess the use of this bio-protective culture to improve the quality and safety of the traditional fermented sausages prepared without antimicrobial additives.

The effectiveness of bio-protective cultures in meat products has been deeply studied against *List. monocytogenes*, but there are other studies that assess the antimicrobial activity of LAB cultures against other food-borne pathogens. Comi et al. (2015) inoculated a mix of bio-protective culture in beef hamburger packaged in modified atmosphere ad stored at refrigerate temperature with the aim to inhibit the growth of *B. thermosphacta* and improve of the microbial and organoleptic qualities of the meat. While Di Gioia et al. (2016) tested the possibility to use two LAB strains (*Ltp. plantarum* PCS20 and *Lactobacillus delbrueckii* DSM20074) as bio-protective cultures against *Clostridium* spp. in pork ground meat designed for fermented salami preparation. In meat fermented foods, *Clostridium* spp. growth can be controlled by the addition of nitrite (Linton et al., 2014). Nevertheless, the growing consumer request to avoid the addition of these additives has led the food industry to consider alternative approaches and the use of bio-protective cultures being one of them. In this work both strains showed anti-clostridia activity *in vitro* (spot agar test), but *Ltp. plantarum* PCS20 was able to effectively survive in ground meat and of performing anti-microbial activity against the target microorganism. Therefore, this work is important in the perspective of reducing or removing the amount of nitrite added into fermented meat products by using bio-protective cultures, as a feasible solution.

Moreover, this approach could be coupled with the application of non-thermal processes, as a strategy to increase the safety of RTE meat products (Bover-Cid et al., 2019).

Finally, it is necessary to assess the impact of these technological strategies taking into consideration the processing conditions and the characteristics of the different final products. Thus, the application of LAB strains as biocontrol agents and the use of more accurate tools can significantly prevent the growth of food-borne pathogens or spoilage microorganisms and contribute to the extension of products shelf-life, without quality loss.

1.3.2 Application in fish and seafood products

Fish and fishery products are considered an important reservoir of nutrient, even if they can be highly susceptible to spoilage (Sheng and Wang, 2021). Many of these products are designed to be consumed as a RTE foods, minimally processed since the consumer demand for the use of mild technologies and naturally preserved foods increases. This food category can be mainly associated with the presence of *List. monocytogenes*, a food-borne pathogen that can cause human listeriosis (EFSA Biohaz, 2018). For this reason, in the last years the research of new alternative strategies has been required. With this aim, LAB strains are considered a promising solution also in fish industry applications (Rathod et al., 2022). Recently, a lot of studies have been focused on the use of these bio-protective cultures, with their competition with spontaneous microbiota as well as their antimicrobial metabolites production, to inhibit food spoilage of pathogens microorganisms in this type of products, improving quality and extending their shelf-life (Table 1.4). In fact, the capacity to produce bacteriocins *in situ* by some LAB strains can represent a feasible strategy to prevent the growth of undesired microorganisms. Moreover, to prolong fresh fish shelf-life other technological processes are increasingly used to slow the microbial degradation. Smoking is one of these techniques: fish products such as salmon and trout are salted and cold-smoked to increase their organoleptic characteristics and shelf-life.

Table 1.4: Application of LAB and their metabolites in fish products preservation (adapted from Rathod et al., 2022).

Bio-protective culture	Product	Note	References
LAB isolated from intestine of *Oreochromis* sp.	*In vitro* analyses	Anti-listerial activity, antagonistic activity	Sanyal and Chatterjee, 2021
Pediococcus pentosaceus LJR1	White leg shrimp	Reduction of List. monocytogenes by 1 log cfu/g on shrimp	Ladha and Jeevaratna, 2020
Lat. sakei CTC494	Ready-to-eat fish products (sliced surimi and tuna paste)	The microbial inhibition by bacteriocin was dependent upon the product	Bolívar et al., 2021
Different selected LAB cultures	Salmon dill gravlax	Antimicrobial activity against spoilage microbiota and *List. monocytogens* showed by *Carnobacterium maltaromaticum* SF1944	Wiernasz et al., 2020
Lat. curvatus, *Carn. maltaromaticum*, and *Lat. sakei* CTC494	Different types of cold-smoked salmon	*Lat. sakei* CTC494 inhibited the growth of *List. monocytogens* and other spoilage microbiota, increasing shelf-life of all three types of smoked salmon; with the other two strains the inhibition of pathogens growth depending on the type of smoked salmon product	Aymerich et al., 2019
Ent. mundtii	Fish paste	*Ent. mundtii* STw38 showed highest activities against Gram-positive bacteria including *List. innocua*	Delcarlo et al., 2019
Combined culture of *Ltp. plantarum* AB-1 and *Lacticaseibacillus casei*	Shrimp (*Litopenaeus vannamei*)	The application of combined LAB cultures significantly reduced spoilage microorganisms, mainly *Shewanella baltica*, and increased the shelf-life of the shrimp.	Li et al., 2019
Combination of *Lactococcus piscium* CNCM I-4031 and *Carn. divergens* V41	Peeled and cooked shrimp (*Penaeus vannamei*)	Shrimp treated with combined cultures had higher sensorial properties and lowered microbial and chemical changes at the end of storage, compared to the treatment with single LAB culture	Saraoui et al., 2017
Six LAB strains with other hurdles	Cod and salmon-based products	Improved sensory properties and reduced microbial and chemical changes in cod and salmon products	Wiernasz et al., 2017

In this context, Saraoui et al. (2017) investigated the combined effect of *Lactococcus piscium* CNCM I-4031 and *Carn. divergens* V41 to improve quality and safety of peeled and cooked shrimp. Compared to samples inoculated with the single LAB strain, the co-culture showed a synergic effect in maintaining the sensory properties of the shrimp product, with the same antimicrobial activity.

More recently, *Lat. sakei* CTC494 strains, that was previously recognised as a starter and bio-protective culture for fermented sausages manufacturing (Ravyts et al., 2008), has been tested on fresh fish products (Costa et al., 2019). In their study this sakacin-producing strain was able to inhibit *List. monocytogenes* growth when simultaneously inoculated in the sample. According to the microbiological results and sensory analyses, an initial inoculation level of 4 log cfu/g for *Lat. sakei* CTC494 could be a suitable strategy to control the safety aspects of fish products, without compromising their sensory quality. Aymerich et al. (2019) also studied the effectiveness of the same bio-protective culture in different types of cold-smoked salmon. *Lat. sakei* CTC494 inhibited the growth of *List. monocytogens* and other spoilage microorganisms, even in samples with a non-

acidic pH and a higher a_w (pH value of 6.0 and a_w of 0.96), with a consequent increasing in shelf-life of all the types of samples analysed. Moreover, in the same study other LAB strains were tested. In particular, *Carnobacterium maltaromaticum* CTC1741, that showed an anti-listerial activity *in vitro* assay, did not exert a significant antimicrobial effect when inoculated into the product except for one type of smoked salmon. Indeed, it is known that food components can affect bacteriocin production and antimicrobial activity of bio-protective cultures. Further research has focused on the assessment of *Lat. sakei* CTC494 against *List. monocytogenes*. In this case, Bolívar et al. (2021) tested this bio-protective culture into two different RTE fish products: surimi-based product and tuna pâté. The inhibiting effect of the selected LAB strain on the pathogen growth was product dependent: in fact, in surimi sample the inhibition of pathogen growth was reached with the maximum cell load of *Lat. sakei* CTC494, while in tuna pâté there was a strong inhibition of *List. monocytogenes* at 2°C and a limitation in its growth at 12°C.

In another study, Delcarlo et al. (2019) reported that, among some selected strains isolated from mussels of the Argentine coast tested into fish pasta, *Ent. mundtii* Stw38 was characterised by a better activity against Gram-positive bacteria, including *List. innocua*, thanks to its higher growth rate and bacteriocin production. Moreover, six different LAB strains were tested in vacuum-packaged salmon gravlax as bio-protective cultures (Wiernasz et al., 2020). Some of them showed a high growth competition and an antimicrobial activity against spoilage microorganisms, but *Carn. maltaromaticum* SF1944 was characterised by the highest anti-listerial activity and the lower organoleptic impact. Finally, Iacumin et al. (2021) also investigated the activity of different LAB strains, including *Lat. sakei* LAK-23 (Sacco srl), against *List. monocytogenes* in cold-smoked sea bass product, RTE foods that could support this food-borne pathogen growth (pH > 6 and a_w > 0.97). Only *Lat. sakei* LAK-23 was able to stop the growth of *List. monocytogenes*, that remained at the initial inoculum level until the end of storage thanks to this bacteriostatic effect. In addition, the activity of this bio-protective strain did not cause any off flavour or off odour in the product.

All these works demonstrated that, overall, bio-protective LAB cultures compete with spoilage or pathogenic microorganisms for nutrition consumption, becoming dominant also due to the production of metabolites with antimicrobial activity against these undesirable microorganisms.

1.3.3 Application in other products categories

Finally, milk and products derived from it can also represented another food category that can support the growth of pathogenic and food spoilage microorganisms. They can be controlled through the application of bio-protective LAB cultures. For example, in fermented dairy products the risk of contamination is lower because of antimicrobial compounds, including organic acids and

bacteriocins, produced by LAB strains (Ağagündüz et al., 2022). Therefore, these cultures have an important role in the dairy industry as they improve the organoleptic qualities of fermented milk products and extend their shelf-life.

The study of Scatossa et al. (2017) showed that the addition of a mixture of *Lacticaseibacillus rhamnosus, Lc. lactis* and *Enterococcus faecium* during cheese manufacture could be resulted in the inhibition of *List. monocytogenes* growth through the production of bacteriocin-like compounds. The potential as bio-protective cultures of other two *Enterococcus* strains, *Ent. faecium* ST88 and *Ent. mundtii* CRL35, was also evaluated against *List. monocytogenes* into fresh Minas cheese (Pingitore et al., 2012).

Finally, Tabanelli et al. (2020) assessed microbial quality and safety of Ricotta filled pasta (Tortelloni), an Italian typical fresh product, through the study of milder heat treatments combined with the addition of two LAB strains as bio-protective cultures, belonging *Lacticaseibacillus paracasei* and *Ltc. rhamnosus* species. The results demonstrated that these LAB had a relevant impact on the microbiota of Tortelloni during storage. Although they were not dominant, their presence reduced the initial microbiota associated with raw materials. Moreover, the use of bio-protective cultures could be considered a helpful strategy to reduce thermal treatments and to better maintain the traditional textural and flavour characteristics of this product.

1.4 Starter cultures: technological advances for improving quality and safety of fermented products

As mentioned above, in the last years the research is focused on bio-protective techniques, in which lactic acid bacteria strains can be employed to reduce microbial degradation. This is possible thanks to their competition with spontaneous microbiota and food-borne pathogens and their ability to produce specific metabolites, such as bacteriocins. In particular, in these studies new LAB strains are evaluated for their safety aspects and antimicrobial properties in order to ensure their safe application.

Furthermore, in food industry LAB strains are introduced in fermentation processes, not only as bio-protective cultures to enhance food safety, but also as starter ones due to their technological features (Cocconcelli and Fontana, 2010). In particular, this strategy was widely used in dairy industry for cheese, yogurt and other fermented products (Aryana and Olson, 2017), while their application is recently introduced in meat industry (Laranjo et al., 2017).

In the past, the earliest productions of fermented foods were based on a spontaneous fermentation achieved by the growth of microorganisms naturally present in the raw material. This process could lead to a poor-quality product, characterised by the risk of the presence of undesirable

microorganisms, such as amino biogenic producer or pathogens, that compromised the safety aspect of these artisanal products (Ojha et al., 2015). For this reason, nowadays, the use of starter cultures has become increasingly necessary to ensure high quality and safety levels in fermented products, with an improvement of their organoleptic characteristics (García-Díez and Saraiva, 2021). This application has allowed the standardisation of products, obtained with a controlled fermentation process, even if this aspect could result in a loss of their typical characteristics, also probably due to the limited number of commercialised strains. With this regard, traditional products can represent an important source of isolation of new LAB strains, considered promising to be employed as starter cultures since they can be well adapted to the food matrix and dominate the environment of these products, preserving their biodiversity and improving their peculiarities.

Generally, commercial starter cultures are composed by a mixture of microorganisms belonging from different species. In particular, staphylococci (*e.g. Staphylococcus xylosus* and *Staphylococcus carnosus*) are added during fermentation to obtain desirable organoleptic properties and maintain physico-chemical characteristics, such as colour formation and its stability. On the other hand, LAB strains (*e.g. Lat. sakei*, *Lat. curvatus* and *Ltp. plantarum*) are used to compete with indigenous microbiota and to control the process by driving a rapid environmentally acidification, which can prevent the growth of undesirable microorganisms. Moreover, these functional cultures can also enhance microbial safety and stability of the products by producing antimicrobial compounds (bacteriocins). For these reasons, the application of these novel bio-protective starter cultures has attracted a great attention in food industry.

1.4.1 Safety risks associated with fermented meat products

Fermented meat products, such as fermented sausages, are manufactured by minced meat and fat, generally mixed with salt, additives and spices, then stuffed into natural or synthetic casings and subjected to a process in which a microbial fermentation occurs, with a consequent pH and a_w decrease. These fermented foods are considered as safe products, thanks to technological hurdles, represented by low values of pH and a_w, presence of salt, nitrites and spices, that can be responsible for the pathogenic and spoilage microorganisms' inhibition (Puolanne and Petaja-Kanninen, 2014).

Among these hurdles, the use of nitrites is explained by the fact that these compounds have both technological and safety advantages. In particular, they can inhibit the growth of some pathogenic microorganisms, especially *Clostridium botulinum*, delay oxidative rancidity and contribute to the formation of the typical cured meat flavour and colour (Sindelar and Milkowski, 2012). Despite the functions exerted and their strictly controlled use in meat (Directive 2006/52/EC), their addition in food raised many concerns related to their potential link with the

production of carcinogenic N-nitroso compounds (nitrosamine) (Bernardo et al., 2021). Thus, the reduction/absence of nitrate, as well as the restriction in fermentation and ripening time or the decrease in salt concentration, are pursued by industries in order to obtain more healthy products. Conversely, this has allowed the potential growth or survival of food-borne pathogens, causing outbreaks (Fraqueza et al., 2020).

In this context, many authors have recognised in their studies the effect of starter cultures, alone or in combination with other techniques, as a key role to guarantee food safety (García-Díez and Saraiva, 2021; Laranjo et al., 2017). However, to select microorganisms to be used as promising starter cultures, it is necessary carry out proper studies firstly regarding their safety aspect, followed by evaluation of their functional and technological properties. Only after these screening to better characterised the new isolated strains, these potential starter cultures could be used in the industrial processes as autochthonous starter cultures to improve the peculiarities of the products. Moreover, it is important also tested and confirmed their performances into real food systems.

1.4.2 Screening of LAB strains regarding their safety aspects to select new candidate to be used as starter cultures

Starter cultures play an essential role in the manufacture of fermented products. In particular, LAB strains are the starter cultures mostly involved because they can be dominant into the microbial consortium of these foods, with an impact on both technological properties and microbial stability of the final product, preventing or controlling microbiological hazard (Laranjo et al., 2019). Therefore, to better select a good strain to be applied in an industrial process, it is important assess its specific performances also linked to the type of product to be obtained.

First of all, LAB strains selected to be new candidates to be starter cultures must be safe. With this regard, it is necessary to evaluate their capacity to produce biogenic amines (BA) and to assess their antibiotic-resistance profile.

1.4.2.1 Biogenic amines

Biogenic amines (BA) are nitrogenous compounds that can be found in fermented foods, affecting their quality and safety. The most common BA detected in these types of products are histamine, tyramine, putrescine, cadaverine and 2-phenylethylamine, derived from free amino acids decarboxylation by microbial metabolism (Ruiz-Capillas and Herrero, 2019). These compounds can cause dose-dependent toxic effect on human health, from allergy symptoms to systemic clinical signs, and for this reason it is important to effectively control their content. Moreover, BA are

thermostable and further processing of foods could not eliminate them once formed. The most dangerous BA are represented by histamine and tyramine, that can cause toxic symptomatology known as "scombroid fish poisoning" and "cheese reaction," respectively (Hungerford, 2010; McCabe-Sellers et al., 2006). The first intoxication is linked to the high presence of its amino acid precursor (histidine) in several seafoods, such as scombroid fishes belonging to the families *Scombridae* (tuna and mackerel) and *Scomberesocidae* (saury), as well as other type of fish (sardines, herring, anchovies, bluefish, marlin). For this reason, the content of histamine in fishery products was regulated by the Regulation EC No. 2073/2005, as reported in Table 1.5 (European Commission, 2005).

Table 1.5: Legal limits for histamine in fishery products placed on the market during their shelf-life (European Commission, 2005).

Food category	Micro-organisms/their toxins, metabolites	Limits	Analytical reference method	Stage where the criterion applies
1.26_Fishery products from fish species associated with a high amount of histidine	Histamine	100-200 mg/kg	HPLC	Products placed on the market during their shelf-life
1.27_Fishery products, except those in food category 1.27a, which have undergone enzyme maturation treatment in brine, manufactured from fish species associated with a high amount of histidine	Histamine	200-400 mg/kg	HPLC	Products placed on the market during their shelf-life
1.27a_Fish sauce produced by fermentation of fishery products	Histamine	400 mg/kg	HPLC	Products placed on the market during their shelf-life

The use of starter cultures can represent a strategy to counter BA formation. Certainly, LAB strains, that are tested to be a possible application as starter cultures in fermentation processes, must not be able to produce these compounds. Many studies have focused on the amino biogenic potential of LAB associated with fermented foods to verify their inability to produce these compounds, since they are considered as the main BA producers in these products (Barbieri et al., 2019). On the other hand, amine-negative LAB strains could reduce the risk associated to the accumulation of BA, limiting the growth of amino biogenic producer microorganisms through their competitive effect against the natural microbiota (Capozzi et al., 2020). Several studies demonstrated the role of LAB starter cultures in reducing the accumulation of BA in meat products (Elias et al., 2018; Lorenzo et al., 2017; Lu et al., 2015). Moreover, the application of strains producing amine-degrading enzymes represent a novel biotechnological alternative to affect BA accumulation (Li and Lu, 2020).

All these information can help to improve the selection and characterisation of strains for further applications as starters or bioprotective cultures, in order to obtain high quality foods with reduced BA content.

1.4.2.2 Assessment of antibiotic-resistance profile

Despite LAB classification as safe microorganisms (GRAS), another issue that can affect them is represented by antibiotic-resistance and its potential horizontal transfer (acquired resistance) to pathogenic microorganisms constitute a risk to be considered (Colautti et al., 2022). According to WHO report (2014), the emerging antibiotic-resistance, as a consequence of environmental changes, is a growing human health concern since many of the available treatment options for common infections could become ineffective.

For this reason, LAB strains selected as potential starter cultures with technological or bio-protective characteristics need to be phenotypically assessed for antibiotic-resistance regarding clinically relevant antibiotics, before their application into food industry (Fraqueza, 2015). In addition, if a resistance profile is observed, it is important to verify at genome level that this characteristic is intrinsic/natural and not transferable, because of the threat to public health represented by microorganisms containing resistance genes in mobile genetic elements (EFSA Panel, 2017). The most common resistance genes detected in LAB isolated from fermented sausages are those related to mobile elements: in particular, encoding for tetracycline resistance (*tet*M, *tet*W, and *tet*S) and for erythromycin resistance (*erm*B and *erm*C). Therefore, the phenotypic expression of these antibiotic-resistance elements is considered a hazard. On the other hand, these microorganisms can be naturally resistant to vancomycin or aminoglycosides, such as neomycin, kanamycin and streptomycin (Fraqueza, 2015).

In this context, several safety assessments have been performed by different authors on LAB strains usually selected as starter cultures, in order to establish safety in their application in food industry. Among them, Comunian et al. (2010) observed a high number of resistant LAB strains isolated from Salame Piacentino, produced in areas where more intensive animal husbandry practices were applied.

1.4.3 Characterisation of LAB strains regarding their technological features

Once LAB strains safety aspects were ensured, it is necessary characterised these microorganisms regarding their technological features to better investigate the possible application as bio-protective starter cultures in food industry. In particular, a promising strain must be characterised by a strong competitiveness nature against the indigenous microorganisms. Moreover,

it must be able to guide a controlled fermentation process with a fast acidification (pH decrease) and to produce several antimicrobial compounds, such as bacteriocins, to inhibit the spoilage microorganisms or food-borne-pathogens growth, with the final aim to standardise products quality and to improve their safety aspects.

1.4.3.1 Competitiveness of starter cultures

One of the main properties of LAB starter cultures is represented by their potential to colonise the meat environment in spite of indigenous microbial community present in raw material, dominating the whole fermentation process. Moreover, these strains must be able to persist during the entire storage period. Fermented meat products represent a food system characterised by a low temperature, high salt concentration, poor nutritional source and these conditions could be difficult for many microorganisms (Laranjo et al., 2017). However, strains of *Lat. sakei*, one of the principal species detected in fermented sausages (Leroy et al., 2013), showed a higher competitiveness, which could probably be explained by their specialised metabolism well adapted to the sausage environment, as in the case of their arginine deiminase (ADI) pathway (Barbieri et al., 2022).

1.4.3.2 Acidification potential

Another important technological property that must characterised a starter culture is represented by its acidification potential. In particular, a guided fermentation with a controlled and standardised process is the principal results to be obtain through the application of LAB starter cultures. In fact, with their growth during fermentation, LAB strains produce organic acids (lactic acid) that are the main responsible for the pH decrease. The level of acidification and the selection of the starter culture depend on the process parameters, as well as on the desired sensorial properties of the product. For example, Northern and Southern Europe fermented sausages production differs in the process set up, that can be characterised by different duration in fermentation or ripening phases, obtained with different setting temperatures. In particular, the selected LAB starter culture can establish the fermentation time, shorter or longer depending on the strain acidification potential, that is linked with its growth capability in specific production process conditions.

However, the acidification process contributes to the formation of the typical organoleptic characteristics of the fermented sausages, on one hand, but on the other to the inhibition of spoilage and food-borne pathogens due to the low pH reached (García-Díez and Saraiva, 2021). In fact, the acid environment can interfere with the maintenance of the structure and functionality of the cell membrane, leading to cell death. Wang et al. (2015) investigated the antimicrobial effect of organic acids against some the main pathogenic microorganisms in food. They demonstrated that the

presence of these compounds contributed to the safety of the products, due to the consequent formation of an adverse environment (low pH value) that interfered with the cell membrane permeability of the target microorganisms. However, it is important underline that in this study the antimicrobial effect of organic acids was assessed by their addition as a "natural additive" and not during fermentation processes in foods. In a real food system, the inhibitory effect of these compounds could be resulted in a synergic action with other metabolites produced by starter cultures and not by the individual activity of each one (Arena et al., 2016).

1.4.3.3 Production of antimicrobial compounds

In addition, as mentioned before, a further feature that can characterise LAB starter cultures is represented by functional aspects. In particular, their possibility to produce specific compounds with an antimicrobial activity against spoilage and food-borne pathogens (reference to Paragraph 1.2). This can allow to find out new LAB strains characterised by both technological and bio-protective properties, that can make them a promising application in food industry in order to obtain high quality products with improved safety and prolonged shelf-life.

1.4.4 Application of starter cultures in meat industry: two case studies

Once the technological and bio-protective properties of LAB strains were demonstrated *in vitro* tests, it was necessary to assess the effectiveness of the same cultures into real food systems since this more complex environment and their characteristics could affect the performances of these promising strains, as well as the competition with wild microorganisms.

Several studies clearly demonstrate that *Lat. sakei* is the predominant species in fermented meat products, and its use as a starter culture for sausage production is widespread (Leroy et al., 2014). However, the addition of starter cultures is necessary but not sufficient to obtain an acceptable product quality level. They must be dominant respect to the indigenous microorganisms present in the raw material, in order to achieve products with the desired characteristics and to guarantee their safety, but their application needs to be adjusted in relation to the entire process parameters. Nevertheless, the use of these starter cultures still followed some empirical operations in which they are considered as an added additive. Conversely, this step is crucial tin order to obtain safe products with desirable organoleptic characteristics (Montanari et al., 2021a). Moreover, the quality of the raw material can play an important role on the result of the final product. In particular, the initial microbial contamination of meat can affect the ability of the selected bacteria to colonise the environment, bringing to safety (BA content) and organoleptic (off-odours) concerns. The success of the competition can depend on the numerical ratio between starter culture added and

microorganisms naturally present, that usually are between 3.2 and 5.3 log cfu/g (Leroy et al., 2014). Commonly, starter cultures are added 10 times more respect to natural microorganisms initially present. In this way it could be avoided an inadequate activity or competitiveness of starter cultures towards the wild microbiota, with consequent organoleptic and safety concerns.

With this regard, Montanari et al. (2021) reported a case study in which the failed competition between the starter cultures, added during manufacturing, and the wild microorganisms present in the raw materials affected the final product characteristics. In particular, this study was about an industrial lot of Ventricina, an Italian long-ripened traditional fermented sausages. The results showed that the raw meat was characterised by a high microbial count and, for this reason, the starter cultures were not able to colonise the environment reducing or inhibiting the growth of the wild LAB microorganisms. The defects observed (including crust formation and BA accumulation) can be associated with an improper fermentation process, despite the use of starter cultures. Therefore, this study demonstrated that starter cultures application needs to be modulated in relation to production parameters to avoid safety and organoleptic concerns.

Conversely, in another study the successful use of starter cultures was underlined (Tabanelli et al., 2022). These strains were added in a meat batter to obtain a controlled fermentation that led to achieve safe fermented sausages with desirable organoleptic characteristics, without the addition of nitrate and nitrite. The entire production process was deeply modified and starter cultures was added at a high concentration about 7 log cfu/g. In addition, different glucose amount was tested to optimise the process. This parameter, in fact, affected the final characteristics of the fermented sausages, with the better results showed when lower sugar concentration was used in the absence of curing salts. Finally, in this study was also performed challenge tests against selected strains of *List. innocua*, *Salmonella enterica* sub. *enterica* and *Cl. botulinum*. Under the adopted conditions, these target microorganisms were unable to grow into the product. As expected, the nitrate and nitrite removal from fermented sausages requires accurate technological changes to guarantee the final quality and safety of the products.

1.4.5 Application of starter cultures in fish products

Fish fermentation is a process particularly common in Southeast Asia, respect to Europe or Africa. These products can be characterised by several differences, such as raw material used, salt and spices added, or temperature and duration of fermentation process (Zang et al., 2020). This process can be coupled with other technologies to enhance also organoleptic characteristic of these products. Generally, these procedures include salting and drying and, sometimes, also marinating and smoking (Xu et al., 2021).

Historically, as well as for meat, fish fermentation was performed at domestic level firstly to preserve this type of food extremely perishable, but a strict control of its quality is not possible. This concern can also affect the safety of these products, caused by the presence of food-borne pathogens or spoilage microorganisms. Nevertheless, the addition of starter cultures is not still widely applied, due to the different fish species used and the variability in the salt concentration and fermentation conditions, that make difficult the research of good strains able to colonise these environments, driving the fermentation process and ensuring safety aspects (Han et al., 2022).

The possible fermented fish products that can be obtained are represented by an entire fermented fish or part of its, pastes and sauces. The main microorganisms detected in these different products belong to LAB and in particular to the genera *Leuconostoc*, *Lactococcus*, *Weissella*, *Pediococcus*. They principal role is the pH reduction as a result of organic acids (lactic and acetic acid) accumulation. The environmentally acidification can determine an antagonistic effect against food-borne pathogens or spoilage microflora, together with the production of antimicrobial compounds (*e.g.* bacteriocins) by LAB strains. Moreover, their use can be represented a strategy to reduce BA accumulation. The use of a mixture of strains belonging to *Lactobacillus, Pediococcus, Lactococcus* as starter cultures was investigated by different studies in fish products, in particular when carbon source (mainly rice) was also added to advantage their competitive behaviour with respect to the indigenous microflora (Zang et al., 2020).

Besides LAB, yeasts belonging to *Saccharomyces*, *Candida*, *Pichia*, *Hanseniaspora* and *Debaryomyces* can be frequently found in fermented fish products. Their proteolytic and lipolytic activity contribute to the final characteristics of the product, influencing both texture and organoleptic features. With this regard, some strains of *Saccharomyces cerevisiae* have been reported to produce in Suan yu alcohols with peculiar aromatic notes, such as 3-methyl-1-butanol, 2-methyl-butanol, 2-methyl-propanol and phenethyl alcohol, starting for the corresponding amino acids valine, leucine, isoleucine and phenylalanine (Wang et al., 2017).

Staphylococci also play important role during fish fermentation with a significant contribution to flavour formation (Xu et al., 2021). Some studies showed that strains belonging to the species *Staph. carnosus* and *Staph. xylosus* was able to reduce histamine accumulation during fermentation of fish sauce and in salted and fermented anchovies (Mah and Hwang 2009; Zaman et al., 2014). However, the organoleptic characteristics of final products are obtained through the succession of these microbial groups that dominate different stages of fermentation. For example, in fish sauces, yeasts are dominant in the first weeks of fermentation but, when the pH drops, their number decrease in favour of LAB growth. Then, other species can develop, promoting the formation of a more complex aroma profile.

1.4.6 Application of starter cultures in dairy products

The main starter cultures used in dairy industry for cheese and yogurt production are represented by LAB, especially belonged to the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus*. Their application allows to control and optimise fermentation processes through a rapid acidification of food matrices (Coelho et al., 2022). Moreover, they can play an important role during products ripening thanks to their proteolytic and lipolytic activities, that also contribute to the peculiar flavour and aroma formation, or the possible production of exopolysaccharides (EPS) to improve the final product texture (Jurášková et al., 2022). The proper selection of starter cultures and the technological characterisation of each strain is necessary to achieve products with reproducible organoleptic and structural properties, reducing changes in quality and microbiological stability observed in dairy products obtained without their addition (Carminati et al., 2010). The application of new autochthonous LAB isolated from traditionally products are also considered regarding their use to improve processing, functional value and safety, as well as preserving the peculiarities of different products (Paulo et al., 2022).

Recently, several LAB strains have been demonstrated an interesting antimicrobial activity against undesirable microorganisms and food-borne pathogens as producers of bio-active compounds with health-promoting effects and a consequent safety improvement and shelf-life extension of products (Coelho et al., 2022). In the dairy industry, the main bacterial pathogens that need to be controlled are represented by *List. monocytogenes*, *Staph. aureus*, *E. coli* and *Salmonella* spp. (Al-Gamal et al., 2019). The effectiveness of LAB strains in the inhibition of pathogenic microorganisms is reported in several studies in cheese foods (Coelho et al., 2014; Hammami et al., 2019; Pingitore et al., 2012; Ribeiro et al., 2016). *Staph. aureus* is a concerning pathogen in cheese manufacturing with special relevance in those products made from raw milk, due to its possible production of toxic compounds that, once formed in food, are extremely difficult to delete. Regarding *Salmonella* spp., in cheese processing, this pathogen can decrease along the ripening and storage periods due to salt concentration, storage temperature and pH, that represent the main barriers for its growth, even if in some cases it can survive into the finished product. Some studies demonstrated that the addition of starter cultures in cheese making improved the decrease of *Salmonella* spp. probably associated to the enhanced effect of the pH by lactic acid production (Silva Ferrari et al., 2016; Terpou et al., 2018). On the other hand, *E. coli* can increase in the first hours of cheese ripening and it is investigated that the use of starter cultures can inhibit its growth (Callon et al., 2016). Its inhibition is possible thanks to the acidification potential of LAB cultures (Fretin et al., 2020), in fact it is demonstrated that that survival of *E. coli* during ripening may be associated to the initial lower microbial load of raw milk (Ioanna et al., 2018).

Yogurt, another common dairy product, is obtained by LAB fermentation of milk, previously subjected to a heat treatment and pasteurisation, through the activity of microorganisms such as *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (Das et al., 2019). This product is considered safe due to hurdles included in the production process, such as heated technologies and low pH resulting from fermentation, that make difficult food-borne pathogens viability. However, also in this case, some undesirable microorganisms (*i.e.* pathogens) can contaminate food matrices after fermentation in acid conditions, probably due to acid gene encoded survival mechanisms (Savran et al., 2018).

Finally, a good microbial quality of milk, a proper thermal treatment, a good hygienic manufacturing practices and a proper starter cultures selection must be necessary to obtain products with a high safety and prolonged shelf-life (Martin et al., 2016).

1.5 Bio-preservation: antimicrobial effects of bio-active natural compounds to enhance food safety and extend shelf-life of food products

The increasing consumer demand for high quality, minimally processed and safe foods, produced without synthetic preservatives, led the research towards new bio-preservation alternative strategies. In the Paragraph 1.3, the application of bio-protective LAB strains was widely discussed, but this approach can be also applied in combination with other techniques, in the context of hurdle technologies. In particular, essential oils (EO) and phenolic extracts (EXT), derived from plants or unconventional vegetal matrices, showed promising applications in food industry to enhance the microbial and chemical (oxidative) stability of products, improving their organoleptic properties as well as prolonging their shelf-life (Bajpai et al., 2012). This is linked to their strong antioxidant effect and antimicrobial activity against spoilage microorganisms or food-borne pathogens, such as *List. monocytogenes* and *Salmonella* spp. (Meenu et al., 2023; Nieto, 2020). A possible mechanism of action of these compounds is characterised by the changing in the structure of the cell membrane of the target microorganism, with a consequent increased in its permeability that could affect some functional properties of the cell (Bajpai et al., 2019). In general, potential mechanisms of EO against microorganisms are showed in Figure 1.2.

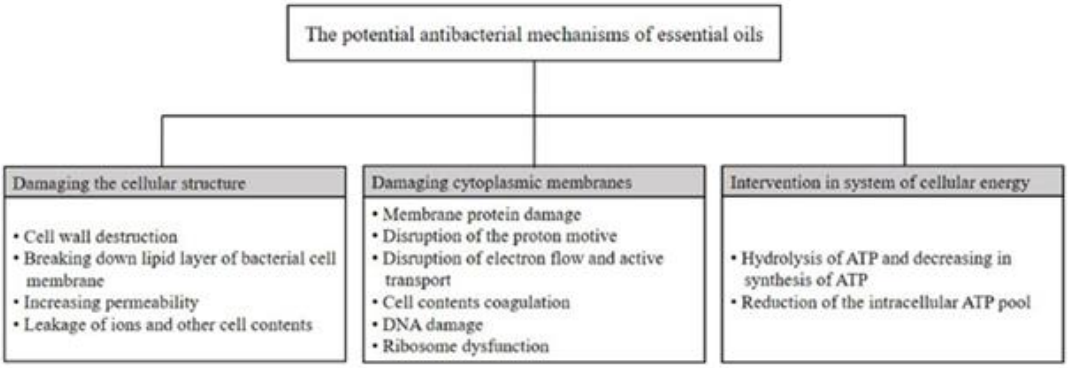


Figure 1.2: Potential mechanisms of EO activity against target microorganisms (Yousefi et al., 2020).

The composition of these natural compounds is characterised by different elements that expressed their bio-protective activity through a synergic effect (Bhavaniramya et al., 2019). EO are complex combinations of several aromatic and volatile compounds with low-molecular-weight obtained from hydro-distillation of several parts of many herbs and spices (flowers, roots, seeds, leaves, etc.) (Chouhan et al., 2017). Their major constituents are phenolic acids, terpenes, aldehydes and flavonoids, produced by plants as secondary metabolites in order to defend them against injuries and parasites, thanks to their antagonist activity.

For centuries, these plants were used in traditional medicine and culinary preparations for improving the organoleptic properties of foods, without really know their antimicrobial potential (Hyldgaard et al., 2012; Sharifi-Rad et al., 2017). Nevertheless, in the last decades the interest regarding bio-active compounds application in food industry increased also in relation to consumers request for "green solutions". This attention was supported also by the feed industry searching for alternatives for antibiotics, whose use has been strongly limited when not banned in animal diets (Omonijo et al., 2018; Sutili et al., 2018).

These plant-derivatives can be applied in food industry in different way: they can be added directly into products during the manufacturing and processing phases, or indirectly into packaging materials, through edible/active films or by incorporation into food coatings. Regarding these latter methods, in their review, Singh et al. (2022) described the use of phenolic compounds into bio-active packaging or edible coatings, novel approaches to improve the oxidative status and antimicrobial properties of food products. These technological strategies can increase the shelf-life of products through the inhibition of undesirable microorganisms and reducing oxidation process. EO can be applied in the same way, but they could negatively affect products organoleptic characteristics and cause their sensory rejection due to an undesirable intense aroma. For this reason, EO are often encapsulated in order to mask flavours, to control their release, but also to improve their stability and solubility (Hao et al., 2021). Agrimonti et al. (2019) demonstrated that cellulosic pads with EO of thyme and oregano showed an antimicrobial activity against spoilage

microorganisms and some common food-borne pathogens (*S. enterica*, *Camp. jejuni* and *Staph. aureus*) in minced beef. Moreover, this meat product packaged with these pads resulted acceptable at sensory test.

Numerous studies reported the exploitation of phenolic extracts and EO preservative properties as antioxidants and antimicrobials strategies, in order to delay lipid oxidation or inhibit spoilage or pathogenic microbial growth, as well as to obtain products with improvement in their textural and flavour properties.

1.5.1 Application of bio-active compounds in meat products preservation

As mentioned before, plant phenolic extracts and EO can be considered a suitable alternative to synthetic preservatives to improve shelf-life and safety of foods, such as meat and RTE meat products (Bajpai et al., 2019). These bio-active compounds are more effective against Gram-positive bacteria, due to their cell membrane structure, so several studies were focused on their application in the presence of this category of microorganisms, that include *List. monocytogenes* (Yousefi et al., 2020). Several studies about the application of different EO to assess their anti-listerial activity in meat products are reported in Table 1.6.

Table 1.6: Application of EO in different meat products to inhibit *List. monocytogenes* growth (adapted from Yousefi et al., 2020).

Essential oil	Meat product	Application	Note	References
Thymus capitata with carvacrol (88.98%)	Minced beef meat	Addition of EO solution to minced beef meat	Application of 0.25 or 1% (v/w) of *T. capitata* EO along with low temperature storage can decrease potential contamination of *List. monocytogenes*	El Abed et al., 2014
Thymus vulgaris L., with linalool (18.18%) and thymol (7.48%); *Rosmarinus officinalis* L., with eucalyptol (13.05%)	Sous vide cook-chill beef	EO added directly on meat	2 log cfu/g reduction of *List. monocytogenes* was occurred in the rosemary-treated samples stored at 2 and 8°C.	Gouveia et al., 2017
Juniperus communis, with α-pinene (47.8%); *Satureja montana*, with carvacrol (30.7%) and thymol (18.0%)	Wine marinated beef	Marination	EO or mixture of EO significantly reduced *List. monocytogenes* in comparison with control sample during 15 days at 4°C. The marinade containing mixture of tested EO exhibited the most pronounced effect.	Vasilijević et al., 2019
Thymus vulgaris L., with thymol (45.9%); *Rosmarinus officinalis* L., with α-pinene (23.98%)	Italian mortadella	Added in the product formulation	Mixture of rosemary and thyme had anti-listerial activity in Italian mortadella: respect to control sample, *List. monocytogenes* was almost lower of 2.29 and 2.79 log cfu/g in samples with 0.025 and 0.05% EO, respectively.	Giarratana et al., 2016
Juniperus communis L., with β-myrcene (14.12%)	Dry fermented sausages	Added in the product formulation	No food-borne pathogens (*E. coli*, *List. monocytogenes*, *Salmonella* spp. and clostridia) were observed in any sample during the storage period.	Tomović et al., 2020
Oregano	Ham slices	In edible films	1.5 log cfu/g reduction of *List. monocytogenes* was observed at the end of storage at 8 and 12°C, while at 4°C a reduction 2.5 log cfu/g was occurred.	Pavli et al., 2019

El Abded et al. (2014) investigated the antimicrobial effect of different concentrations of *Thymus capitata* EO against *List. monocytogenes* ATCC 19118 into minced beef meat. As expected, with the increasing in EO amount there was a gradual decrease in target microorganism detection, respect to the control. In another study, the effect of thyme and rosemary EO (0.025 and 0.05%) against a mixture of three strains of *List. monocytogenes* was tested in Italian mortadella product (Giarratana et al., 2016). The results underlined the fact that the added mixture showed a bacteriostatic effect against this pathogen, that was significantly inhibited in the presence of both tested EO concentrations. In fact, from an initial load level of 2.50 cfu/g, target strains increased to 5.31, 3.01 and 2.52 log cfu/g in control, 0.025% EO-treated and 0.05% EO-treated samples, respectively. Giarratana et al. (2016) also studied the effect of LAB growth and the consequent pH reduction on antimicrobial activity of these EO against *List. monocytogenes*. It is known that pH can affect the effectiveness of these bio-active compounds, that can more easily penetrate the lipid part of the cell membrane enhancing their antimicrobial activity in the presence of low pH values (Brut, 2004). Similarly, Gouveia et al. (2017) assessed the antagonistic effect of rosemary (*Rosmarinus officinalis* L.) and thyme (*Thymus vulgaris* L.) against *List. monocytogenes* ATCC 679 in sous vide cook-chill beef, stored at refrigerated or abusive temperature. The results showed that a reduction of the target microorganism of about 2 log cfu/g occurred in the sample added with rosemary EO at both temperatures tested. The lower antimicrobial effect of thyme EO could be attributed to the low concentrations of thymol in the *T. vulgaris* chemotype that was considered in this study (Gouveia et al., 2017). On the other hand, a higher antimicrobial activity of thyme EO against *List. monocytogenes* in minced meat stored at 4°C was reported by Pesavento et al. (2015), due to the high content of the main antimicrobial constituents of this plan, *i.e.* p-cymene (47.9%) and thymol (43.1%). In another study the synergic effect of soy sauce and teriyaki sauce with carvacrol or thymol (0.3 and 0.5%) against *List. monocytogenes* in marinated beef was evaluated (Moon et al., 2017). This pathogen was not inhibited by the presence of teriyaki sauce alone, while it was inactivated by the addition of 0.5% carvacrol or thymol in combination with the sauce after 7 days of storage.

Moreover, it was reported by several studies that clove and cinnamon EO could be effective against *List. monocytogenes* and in prolonging shelf-life of meat products (Khaleque eta l., 2016; Mytle et al., 2006). The antimicrobial effect of clove is related to eugenol, that can cause the deterioration of the cells wall and their lysis, while the activity of cinnamon EO is linked to the presence of cinnamaldehyde, limonene and eugenol compounds (Calo et al., 2015; Vergis et al., 2015).

In their study, Pavli et al. (2019) demonstrated that the addition of oregano EO into sodium alginate edible films in ham slices led to a 1.5 log cfu/g reduction of *List. monocytogenes* after 40 days of storage at 8°C, while at 4°C this decrease was about 2.5 log cfu/g. Moreover, the combination with other technologies, such as high hydrostatic pressure, significantly increased the inhibition of this target microorganism (Pavli et al., 2019). The oregano EO effect was also studied in RTE smoked turkey meat, packaged in modified atmosphere, that highlighted an antagonist activity against *List. monocytogenes* (Mahgoub et al., 2019). The antimicrobial activity of oregano EO, that was expressed by the presence of a-terpinene, p-cymene, carvacrol and thymol components, was already assessed to control *List. monocytogenes* growth in other previously studies (Dussault et al., 2014; Menezes et al., 2018). In particular, thymol and carvacrol are widely characterised by their antimicrobial activity. The first can cause the degradation of the outer membrane of target microorganisms with an increase in its permeability, while the second can induce changing in the fatty acid profiles and the structure of the cell membrane. Moreover, it can affect the synthesis of flagellin and reduce bacterial motility, as a consequence (Carramiñana et al., 2008; Khorshidian et al., 2018). In one of these previously studies, Carramiñana et al. (2008) tested the effect of savory (*Satureja montana*), thyme (*T. vulgaris*) and rosemary (*R. officinalis*) EO against *List. monocytogenes* in pork meat. They demonstrated that the first two EO were able to efficiently inhibit the growth of the target microorganism, while induced no significant antagonistic effect. This low antimicrobial activity could be attributed to the absence of the carvacrol and thymol compounds in the tested rosemary EO.

In addition, other studies were performed to evaluate the bio-active compounds effect against different food-borne pathogens, such as *Staph. aureus* and *Salmonella* (Table 1.7).

Table 1.7: Application of EO in different meat products to inhibit *Staph. aureus* and *Salmonella* growth (adapted from Oliveira et al., 2018).

Essential oil	Meat product	Application	Target microorganisms	Note	References
Oregano (*Coridothymus capitatus*) with carvacrol (74.87%) and thyme (*Thymus vulgaris*) with β-linalool (79.17%)	Tunisian dry fermented poultry meat sausage	Addition of 0.25% (v/v) oregano and 0.25% (v/v) thyme	*Staph. aureus*	*Staph. aureus* decreased during the ripening phase in sausages added with oregano and thyme EO.	El Adab and Hassouna, 2016
Thyme (*Thymbra spicata*)	Turkish dryfermented sausage (*sucuk*)	Addition of 1.5 g/kg of thyme EO	*Staph. aureus*	*Staph. aureus* decreased almost 4 log cfu/g in *sucuks* with EO after ripening and it was inactivated after 17 days of storage by adding EO.	Erkmen, 2009
Basil (*Ocimum basilicum*) with linalool (71.01%)	Italian-type sausage	Addition different EO concentrations: standard, 0.19, 0.38 and 0.75 mg/g	*Staph. aureus*	Reduction of only 0.4 log cfu/g. until day 14 at EO concentration of 0.75 mg/g.	Gaio et al., 2015
Bay (*Laurus nobilis* L.) with eucaliptol (58.20%), garlic (*Allium sativum* L.) with diallyl trisulfide (33.82%) and diallyl disulfide (18.86%), nutmeg (*Myristica fragans*) with myristicin (43.35%), oregano (*Origanum vulgare*) with thymol (93.34%), rosemary (*Rosmarinus officinalis* L.) with camphor (22.4%) and thyme (*Thymus capitatus*) with thymol (93.94%)	Portuguese dry cured sausage (Chouriço de Vinho)	Addition of EO at two concentrations: 0.05% and 0.005%	*Staph. aureus*, *Salmonella* spp., *List. monocytogenes*	*Staph. aureus* was still presented after 21 days of ripening. After 3 days *Salmonella* spp. was undetectable in the sample with 0.05% of garlic and oregano EO. *List. monocytogenes* weas not detected after 15 days of ripening.	García-Díez et al., 2016

In particular, *Staph. aureus* was significantly reduced during the ripening phase with 0.25% (v/v) oregano and thyme EO in Tunisian fermented poultry sausages (El Adab and Hassouna, 2016). Gaio et al. (2015) evaluated the antimicrobial activity of different concentration of basil EO against *Staph. aureus* in Italian fermented sausages, even if in this trial only a reduction of 0.4 log cfu/g was observed after 14 days with a EO concentration of about 0.75 mg/g. This food-borne pathogen was inhibited at the initial period of ripening, but this was not enough to control its growth until the end of the process.

Finally, the antimicrobial activity of several EO were evaluated against *Staph. aureus*, *Salmonella* spp. and *List. monocytogenes* in a Portuguese fermented sausage (Chouriço de Vinho) (García-Díez et al., 2016). They tested the most effective EO, that included bay, garlic, nutmeg, oregano, rosemary and thyme, identified through a previously disk diffusion assay, at two different concentrations: 0.005% and 0.05%. *Staph. aureus* was the most resistant food-borne pathogen tested. On the contrary, after a smoking treatment, *Salmonella* decreased about 2 log cfu/g and then it was not detectable in the product in the presence of 0.05% of bay, oregano or thyme EO after 12 days of ripening. Furthermore, this microorganism was also under the detection limit at thyme concentration of 0.005%. In their study, García-Díez et al. (2016) tested these bio-active

compounds also against *List. monocytogenes* and the results showed an antimicrobial effect when this pathogen was exposed to 0.005% of oregano, rosemary and nutmeg EO.

In these studies pathogens behaviour changed considerably due to product differences and the composition of natural tested compounds. In general, the microbial susceptibility to the EO antimicrobial effect increased with a decrease in the environmental pH and storage temperature, or in the case high concentrations of them were used. In addition, a combined use of these antimicrobial compounds can induce an enhanced inhibitory effect against food-borne pathogens, thanks to a possible synergic behaviour. However, the organoleptic impact of EO application can negatively affect the sensory properties of the products. Several works underline the fact that higher concentration of EO in food matrices were necessary to obtain the same effect as that achieved in culture media (Burt, 2004; Busatta et al., 2008). This can limit their application in food industry and a strategy to overcome these issues can be represented by using lower concentrations in combination with other bio-preservation technologies, such as the addition of bio-protective starter cultures. Another variable is represented by the composition of EO and the extraction yield, that can be affected by different things: the plants species or their geographic origin, the climate and the soil composition, the part of the plan used for the extraction and the type of extraction, that need to be optimised (Calo et al., 2015).

1.5.2 Application of bio-active compounds in fish products preservation

Bio-preservative effects of several plant extracts and their EO as natural antioxidants and antimicrobials strategies to improve the quality of food products and extend their shelf-life by inhibiting spoilage or pathogenic microorganisms and delaying oxidative processes also interested the fish industry and was reviewed by numerous studies, reported in Table 1.8 (Mei et al., 2019; Rathod et al., 2021; Vijayan et al., 2021). Different pathogenic microorganisms can be present into raw fish material, including *Vibrio* spp., *Aeromonas hydrophila*, *Salmonella* spp., *E. coli*, *Clostridium perfringens* and *List. monocytogenes*, against which the main bio-active compounds applied in seafood industry are oregano, rosemary, thyme, laurel, sage, cinnamon, clove and basil.

Table 1.8: Application of EO in different fish products.

Essential oil	Fish product	Application	Note	References
Oregano and thyme	Grass carp (*Ctenopharyngodon idellus*)	Immersion in EO emulsions (0.1% v/v) for 30 min	EO treatment was effective in inhibiting microbial growth, delaying lipid oxidation and the product shelf-life was extended for 2 days.	Huang et al., 2018
Oregano	Salmon (*Salmo salar*) fillets	Immersion in marinade with EO (1% w/w) for 2 min	The microbial shelf-life of fillets was prolonged. Total aerobic psychrotrophs in samples were reduced after 3 days.	Van Haute et al., 2016
Rosemary	Atlantic salmon (*Salmo salar*)	0.3% EO was added on fillet surface	Significant reduction of *List. monocytogenes* and *Salmonela* Enteridis	Tosun et al., 2017
Rosemary and basil	Rainbow trout (*Oncorhynchus mykiss*) fillets cooked with sous-vide	Addition of EO (100 µl)	The sous-vide packaging in association with EO showed good results in product quality preservation and inhibition of *List. monocytogenes*.	Özturk et al., 2021
Laurel	Rainbow trout (*Oncorhynchus mykiss*) fillets	Nanoemulsions with EO (3.63 and 5.50%) applied onto the surface	Treatment with nanoemulsions showed effective antimicrobial activity on some gram-negative bacteria mostly found in fish fillets.	Meral et al., 2019

Huang et al. (2018) studied the effects of oregano (carvacrol 88.6%), thyme (thymol 72.5%) and star anise (trans-anethole 90.3%) EO on grass carp fillets. The product was immersed into these bio-active compounds and the results showed a reduction in *Aeromonas* and *Shewanella* growth, allowing a two-day increase of shelf-life. Similar results were obtained in the same fish product by using cinnamon EO, derived from bark (Huang et al. 2017). The addition of cinnamon EO into common carp products was responsible for the inhibition of the main spoilage microorganisms and the consequent reduction of the putrescine and cadaverine accumulation (Zhang et al., 2017a).

Other works demonstrated the bio-preservation effect of chitosan-essential oil coatings, treated with lemon and thyme, garlic, clove, cinnamon and lemon grass, onto grass carp products with an improvement in their shelf-life (Cai et al. 2018; Wang et al. 2018; Yu et al. 2017). Moreover, Bahramian et al. (2018) demonstrated that encapsulated *Zataria multiflora* EO was effective in prolonging the shelf-life of rainbow trout, without compromising the organoleptic properties. The organoleptic quality of the same product was enhanced also in Özogul et al. (2017) study, with addition of thyme (71.5% of carvacrol) and rosemary (52.2% 1,8-cineol) EO. The application of this strategy can positively influence the effect of EO antimicrobial activity, by increasing their stability and preventing the interaction with food components, as well as affecting less the organoleptic property of products (Hassoun and Coban, 2017).

Furthermore, Bensid et al. (2014) proposed to directly add plant extracts, in particular thyme (0.04% w/v) and oregano (0.03% w/v), into storing ice used for anchovies, obtaining a shelf-life extension from 5 to 12 days.

1.5.3 Application of bio-active compounds in dairy products preservation

Many dairy products are highly perishable foods, that can be deteriorate within days of their production. To ensure safety to these products, the use of natural preservatives with their bio-active compounds is necessary. In this context, plant derivatives and spices added in dairy products can improve the safety of these foods, prolonging their shelf-life and enhancing their organoleptic properties (Barak and Mudgil, 2023).

Plant derivatives can find their application in cheese manufacturing as antioxidant, antimicrobial and flavouring agents and to improve the stability of these products (Ritota and Manzi, 2020). Several studies were performed in order to assess the antioxidant and antimicrobial activity against the main food-borne pathogens in dairy products. *Nigella sativa* EO was tested against *Staph. aureus*, *S. enteritidis*, *E. coli* and *List. monocytogenes* and to improve storage stability of Domiati cheese. The results reported that the addition of 0.2% EO improved organoleptic and sensory characteristics of cheese and a decrease in food-borne pathogens growth was achieved (Mahgoub et al., 2013).

The effect of cumin, rosemary and thyme EO was studied into soft cheese by El-Kholy and Aamer (2017). They reported that the addition of these EO resulted in an increased antioxidant activity that promoted the extension of product shelf-life. The antimicrobial activity of other plant extracts, included clove, cinnamon stick, grape seed oregano and pomegranate peel, was evaluated in cheese stored at room temperature against the growth of *S. enterica*, *Staph. aureus* and *List. monocytogenes*, that were inhibited by all selected compounds (Bin et al., 2011). Moreover, the stability of cheese against lipid oxidation by their addition was improved.

In another study, Mohamed et al. (2018) demonstrated that the use of *Moringa oleifera* extracts into cream cheese extend its shelf-life up to four weeks and also increased the total phenol content and antioxidant activity of the final product. Total phenolics, antioxidant and antimicrobial activities of herby cheese obtained from sheep milk were enhanced when *Allium vineale* L., *Chaerophyllum macropodum* Boiss. and *Ferula rigidula* DC. were added (Kose and Ocak, 2020).

Finally, Yeriikaya et al. (2021) studied Mozzarella cheeses added with rosemary (*Rosmarinus officinalis* L.), basil (*Ocimum basilicum* L.), peppermint (*Mentha piperita* L.) and oregano (*Origanum onites* L.) aromatic plants. The products showed an increasing into antioxidant

properties and exhibited antimicrobial activities against *E. coli*, *List. monocytogenes*, *Ent. faecalis*, *Staph. aureus* and *B. cereus*.

These studies underlined the fact that these plant derivatives can be considered an interesting strategy, as an alternative to synthetic preservative, for industrial applications.

Moreover, these bio-active compounds can be used in combination with the addition of starter cultures. With this regard, the combination of thymus EO with *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. c*remoris* starter cultures to inhibit *Staph. aureus* was studied (Carvalho et al., 2015). Recently, the synergic effect of EO and starter cultures to control *E. coli* was also studied. In particular, the combination of *Zataria multiflora* EO and *Lactobacillus acidophilus* LA-5 reduced the growth rate of this target microorganism (Mehdizadeh et al., 2018), while the application of the same LAB strain with oregano and rosemary EO totally inhibited the E. coli growth (Diniz-Silva et al., 2020).

1.6 Antioxidant and antimicrobial potential of unconventional matrices affected by drying and extraction methods

1.6.1 Recover of plant by-products to obtain added-value extracts to be applicated into food industry

With the increase in world population and in food products request, a huge amount of biomass (billions of metric tons) is generated every year from the agricultural industry worldwide. The consequent high volume of agro-food industry wastes attracts increasing social, political, and scientific attention at national and international level (Galanakis, 2015).

Agro-food residues can result from non-edible plant parts (*e.g.* leaves), generated after harvesting or during post-harvest process and food processing (Santana-Méridas et al., 2012). As example, about 38% of food by-products occur during food processing, caused by microbial degradation that can result in adverse effect on the environment and human health, high costs for the waste treatment and negative impact in agricultural and economy sector (Helkar et al., 2016).

These by-products are rich in biomolecules with high relevance to the food industry, as well as, pharmaceutical and cosmetic one. In fact, they contain high concentrations of micronutrients (vitamins and minerals), fibres and other secondary phytochemicals, such as phenolic compounds, carotenoids and tocopherols. Among them, a great attention is focused on phenolic compounds, which possess antimicrobial and antioxidant activities and promote benefits for human health, *e.g.* anti-inflammatory and anti-diabetic activities and reduce risk factors of cardiovascular diseases (Martín-García et al., 2020). For this reason and due to the increasing in the consumers demand for

preservatives-free foods, plant by-products can be recovered and reused to obtain added-value extracts to be used as a supplement to produce functional foods (Ayala-Zavala et al., 2011).

Olives are considered a vegetal matrix of a great socio-economical interest and, as a result of pruning, olive leaves represented one of the principal by-products discarded during olive oil production (Santana-Méridas et al., 2012; Talhaoui et al., 2015). This vegetal matrix is characterised by the presence of different classes of phenolic compounds, including simple phenols, flavonoids (luteolin 7-O-glucoside and rutin), oleuropein and hydroxytyrosol (Quirantes-Piné, et al., 2013). Bio-active compounds in the composition of olive leaves extracts make them a potential use as natural additives (Sánchez-Gutiérrez et al., 2021).

Moreover, tropical fruits plant, such as avocado, cherimoya and guava arise a huge amount of by-products and wastes, as another potential source of phenolic compounds. In fact, avocado peel and seed contain great amounts of phenolic compounds and show a higher antioxidant activity (López-Cobo et al., 2016). Similarly, guava leaves are characterised by flavonoids (guajaverin, avicularin and procyanidin B), that can reduce glycemia and insulin resistance (Diaz de Cerio et al., 2016), while cherimoya leaves demonstrate higher antioxidant potential due to the presence of a high concentration of flavonoids (Albuquerque et al., 2016; Diaz de Cerio et al., 2018; Mannino et al., 2020).

In addition, the extracts derived from these agro-food by-products have a demonstrated antimicrobial activity that suggest their potential application as antimicrobials to control food-borne pathogens (*List. monocytogenes*, *E. coli* O157:H7 and *S. enterica*) and in active packaging production or as food ingredients (Al_husnan and Alkahtani, 2016; Biswas et al., 2013; Liu et al., 2017; Rodríguez-Carpena et al., 2011).

However, many factors, including harvest time, cultivation area and agronomical conditions, as well as drying and extraction methods and their standardisation, can affect the phenolic composition of plant residues (Brahmi et al., 2012; Rafiee et al., 2011). The extraction process is an important step in order to obtain a high phenolic recovery from plant matrices (Azmir et al., 2013; Jahromi, 2019). Conventional extraction techniques, such as heating, refluxing or using Soxhlet apparatus could induce oxidation or hydrolysis of phenolic compounds, while maceration requires high volumes of solvents, long extraction times and possesses low selectivity, low reproductive rates and low efficiency (Cifá et al., 2018).

However, in recent years, the developing of green extraction protocol for the recovery of bio-active compounds become crucial for the sustainable use of these by-products in food industry. The application of natural bio-active compounds (antioxidants and antimicrobials) could be a possible solution to guarantee food security and the environmental sustainability of agro-food

systems, in order to valorise food by-products and extend perishable foods shelf-life, maintaining the food nutritional and sensorial properties and reducing microbiological risks.

1.6.2 Marine algae: a*n interesting source of bio-active compounds with a promising application as food ingredients*

It is well known that marine macroalgae have strong antioxidant, antimicrobial, anti-inflammatory and anticancer properties (Pérez et al., 2016; Wijesinghe and Jeon, 2012). Phenolic compounds (phenolic acids, flavonoids, and tannins) are the most important bio-active compounds found in macroalgae (Biris-Dorhoi et al., 2020). They are the primary components of algae cell walls and are produced as a response to harsh environmental conditions. Moreover, they have a role as a chemical defence against herbivores, bacteria, and fouling organisms (Bernardini et al., 2018; Generalić Mekinić et al., 2019).

Studies on bio-active components from algae have intensified in the last years and most of the focus has been on the identification of new components. Phenolic compounds, beside to improve consumers health (cardioprotective, antihypertensive and anti-inflammatory properties), can exert antioxidant and antimicrobial features (Martín-García et al., 2020). To date, it has been reported that seaweeds are a rich source of catechins, rutin, quercetin, hesperidin and other flavonoids (Cotas et al., 2020). Strong antioxidant properties of phenolics make them a functional ingredient for possible applications in food products, also in order to satisfy the increased interest in natural antioxidants as a replacement for synthetic ones (Kranl, et al., 2005).

The selection of the drying and extraction method is a key factor that influences the phytochemical content of algae samples. It has been reported that many parameters, such as temperature, drying time, and UVA-UVB light can affect the phytochemical content and antioxidant potential of vegetal matrix (Ling et al., 2015). Before extraction, macroalgae are traditionally sun-dried, shade-dried, or oven-dried, but for the previously mentioned reasons, protocols of drying and extraction methods require adaptation dependent on the type of samples. In fact, as low temperature prevents the degradation of heat-sensitive compounds, freeze-drying, a method that is performed under vacuum, has been suggested to prevent the oxidation reactions of functional ingredients (Amorim et al., 2020).

Recently, researchers studied alternative methods to extract phenols from macroalgae. In particular, ultrasound-assisted extraction (UAE) and microwave assisted extraction (MAE) have been investigated. These novel methods are considered green methods since they reduce extraction time, decrease high solvent consumption and increase the yield of targeted compounds, as compared with conventional ones, such as maceration or shaking (Phasanasophon and Kim, 2018).

In addition, hydro-alcoholic mixtures could be the best suitable solvent for the extraction of phenolics compounds, being also acceptable for food applications (Medina-Torres et al., 2017). This solvent mixture is characterised by a synergistic effect: water acts as a swelling agent of the matrix, while ethanol promotes the breaking of bonds between solutes and the matrix (Sahin and Samlı et al., 2013). However, extraction parameters, solid-to-solvent ratio, and solvent choices need to be optimised and investigated to find conditions that would yield the highest amount of targeted compounds and preserve their biological activity.

In the last few years, several studies have been focused on the analysis of the application of different extraction methods and different solvents to obtain bio-active compounds with specific biological activity from *Padina pavonica* algae (Table 1.9).

Moreover, the effect of seasonal harvest can also affect the extract composition and the antioxidant and antimicrobial activities of these vegetal matrices, as demonstrated by different studies (Čagalj et al., 2022).

Table 1.9: Research studies on different extraction methods and solvents to yield specific biological compound/activity of *Padina pavonica* (Čagalj et al., 2021).

Extraction	Solvent	Bio-active compounds/activity	References
MAE	Ethanol and methanol 80%	Antibacterial activity	Alghazeer et al., 2017
Centrifuge at 37°C for 2.5 h	Ethanol 96%	Phenolic compounds	Mannino et al., 2017
Stirring for 12 h	Methanol and dichloromethane	Antioxidant activity, cytoprotective-potential	Pinteus et al., 2017
Soxhlet extraction	Acetone	Pro-apoptotic or pro-osteogenic activity, antioxidant activity	Bernardini et al., 2018; Minetti et al., 2019
MAE, pressurised liquid extraction (PLE), supercritical fluid extraction (SFE), electroporation extraction	Petroleum ether, ethanol, ethyl acetate and water	Anti-hyaluronidase activity	Fayad et al., 2017
Extraction with reflux condenser at 100°C for 2 h under reduced pressure	Acetone and water	Antioxidant activity, antimicrobial activity	Hlila et al., 2017
Shaking extraction for 72 h	Acetone, ethanol and methanol 80% and water	Antioxidant activity, antidiabetic activity	Ismail et al., 2020
Maceration at room temperature twice for 24 h	Hexane, ethyl acetate and methanol	Antiparasitic activity, antioxidant activity	Chiboub et al., 2017
Supported liquid extraction (SLE) for 30 min	Ethanol 50%	Antioxidant activity, anti-inflammatory activity, antinociceptive activity	Abdelhamid et al., 2018
Percolation at room temperature for 48 h	Ethanol 95%	Antimicrobial activity, antioxidant activity, anticancer activity	Al-Enazi et al., 2018

1.6.3 New extraction techniques for phenolic compounds recovery from plant by-products

Antioxidant and antimicrobial activities of agro-food residues extracts are related to the phenolic compounds that are present in these extracts and their quantitative recovery represents a crucial point.

Toxic effects of solvents and other problems are associated with conventional extraction methods, such as solid-liquid extraction and maceration. For this reason, alternative extraction techniques of phenolic compounds are needed. Nowadays, new sustainable alternative methods are characterised by shorten extraction times and reducing in toxic solvent consumption (Khoddami et al., 2013; Putnik et al., 2018). In particular, microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE) and pressurised-assisted extraction (PLE) are characterised by an increasing attention, as these techniques have shown to be efficient in the recovery of phenolic compounds from plants (Zhang et al., 2018b). MAE is a thermal technology that is widely used for phenolic extraction (Zhang et al., 2011), but this can damage thermally unstable bio-active compounds, resulting in more expensive and more difficult effectiveness. Because of that, non-thermal technologies (*i.e.* ultrasounds) can represent a solution to obtain safe and shelf-stable plant-based extracts, with an increase in phytochemicals content, compared to thermal techniques.

In particular, UAE is a green alternative method, that requires a lower investment of solvent, energy and time. Moreover, it is easy to handle and represents a feasible technological option for industrial scale-up, with high reproducibility, high extraction yields and environmental sustainability (Irakli et al., 2018; Medina-Torres et al., 2017; Zhao et al., 2021). Briefly, this technique consists in the propagation of the ultrasonic waves through a liquid medium, which causes damage of cell walls, resulting in an improvement in solvent penetration and the subsequent release of the content of phenolic compounds (Mohamed Ahmed, et al., 2020; Zhang et al., 2018b). Two types of devices are used in ultrasound-assisted extraction: an ultrasonic bath and ultrasonic probe (sonotrode) equipment (Chemat et al., 2017). However, the sonotrode system is more powerful in comparison with the ultrasonic bath, because the ultrasonic intensity is delivered through a smaller surface (the tip of the probe) (Martín-García et al., 2022a,b; Sukor et al., 2018). With UAE, the most influential factors on the yield of phenolic compounds are the solid/liquid ratio, extraction time and solvent concentration (Elshreef et al., 2021).

Moreover, pre-treatments (enzymatical treatment, fermentation, etc.) are generally applicated onto these new assisted extraction methods with the aim to increase the extraction yields and improve the process kinetics of the extraction of phenolic compounds (Gil-Chávez et al., 2013). In fact, several enzymes (cellulase, β-glucosidase, xylanase, pectinase, etc.) can enhance the cell wall degradation, facilitating the phenolic compounds release. Similarly, microbial enzymes

produced during fermentation can increase the yield and change the profile of phenolic compounds, as a consequence of the degradation of the cell wall structure (Dey et al., 2016).

Fermentation processes can be described by two different methods: submerged fermentation (SmF), which is based on microbial growth into a defined medium, containing nutrients, or solid-state fermentation (SSF), characterised by the growth of selected on solid particles in the absence of water (Starzyńska-Janiszewska et al., 2022).

Finally, purification of phenolic extracts can be performed by several green processes, such as membrane filtration and resin purification, that need to be defined through an appropriated selection of membrane typology, as well as an optimisation of parameter conditions, depending on the specific plant by-products (Cassano et al., 2018). Their high efficiency, lower cost and simpleness make them an interesting commercial strategy to the recovery of phenolic compounds (Soto et al., 2011).

Chapter 2:

General aim

2.1 General aim of PhD thesis

Global projections show that food demand will increase by 35% by 2030, due to the substantial population growth, urbanisation and economic development of emerging countries.

The possible solutions to this evidence will necessarily have to go through an increase in production yields, but also through the reduction of food waste in productive systems and supply chains, with an integrated perspective of sustainability (Hoff, 2011). On the other hand, the Food Safety Committee of FAO (2015) indicates the degradation of the product, especially for fresh and minimally processed foods, with the consequent reduction in microbiological quality, as the main factor responsible for waste increase at all stages of the production. In addition, in recent years, also given the different consumption habits and changes in lifestyles, minimally processed foods have been involved in several outbreaks, being the source of food-borne pathogenic bacteria. In particular, food safety can be a recurrent concern in productions obtained in traditional and local small-scale enterprises, lacking an efficient system of traceability and product quality standardisation.

For these reasons, the development of sustainable approaches to assure safety, quality and shelf-life of both fresh and fermented products is a crucial aspect of pursuing production efficiency ensuring high food levels and dietary habits. These goals can be achieved through an optimisation of biotechnological tools, affordable also by local producers.

In this perspective, the application of new microbial cultures endowed with peculiar features and natural bio-active compounds could provide innovative biotechnological tools to meet consumer demand for safe minimally processed products, reducing small-scale traditional production safety concerns, valorising sustainable sources and by-products and help to guarantee environmental sustainability of agro-food systems.

The high quantity of artisanal spontaneous fermented sausages from different Mediterranean countries represents a relatively unexplored reservoirs of microbial biodiversity, within which it is possible to find new microorganisms with relevant biotechnological properties, both for fermentation and bio-protection purposes. The exploitation of the important microbial biodiversity of some spontaneously traditional fermented products by bioprospecting of strains isolated from these environments can be an effective strategy to find strains of industrial importance and, at the same time, to fill the gap between the laboratory results and efficacy in the production field for bio-protective and starter cultures.

In fact, the importance of isolating and selecting biocontrol agents or starter cultures from the same environment where they will be reintroduced has been demonstrated, due to strains

adaptation in specific ecological niches and their growth, colonisation and antagonistic capacities, with enhanced potential as biocontrol culture (Siroli et al., 2015).

Fermentation agents, such as LAB, used in numerous products due to their technological properties, can also carry out bio-protection activities, due to their competition with spontaneous microbiota by the production of specific metabolites (*i.e.* bacteriocins, organic acids, etc.), assuring food safety and extending product shelf-life while maintaining the food nutritional and sensorial properties (Chikindas et al., 2018; Elsser-Gravesen and Elsser-Gravesen, 2014). However, the availability of already commercialised starter or bioprotective cultures is limited to a small number of strains that are not representative of the natural microbial biodiversity of meat products. For this reason, the use of commercial starter cultures can enhance product safety, but, on the other hand, can cause a standardisation of the product, with a loss in their recognisability and typicity linked to local cultural heritage. It is well known that the great variability characterising traditional fermented sausages depends also on the sensory properties originally determined by a microbiota typical of a geographic area, as well as of a peculiar productive process, and industrial standardisation can represent a limitation for the local production of regional products.

This is the reason why in the last years an increasing interest has been addressed to the isolation of new autochthonous starter cultures or bioprotective agents, endowed with technological and antimicrobial features, that can be potentially used in meat products due to their ability to confer specific peculiarity to the productions (Bassi et al., 2015; dos Santos Cruxen et al., 2019; Franciosa et al., 2022; Talon et al., 2008).

In addition to the possible application of bio-protective cultures, the use of natural molecules with a broad spectrum of antimicrobial potential, have been proposed to meet the consumer demand for safe foods without chemical additives. It is well known that many plants can be an important source of bio-active molecules and substances generally recognised as safe (GRAS) and widely accepted by consumers. In fact, many plant extracts, essential oils (EO) or their constituents can contain molecules with a relevant antimicrobial activity and have been proposed as potential preservative ingredients in several foods or as antimicrobial agents (Prakash et al., 2015; Vergis et al., 2015). The search for new sources of natural compounds can lead to the exploitation of under-valorised plant matrices and by-products of agro-food system. Among these sources, Mediterranean maquis is a natural ecosystem with many aromatic plants characterised by high biodiversity and many species able to produce antimicrobial substances. The investigation of the potential exploitation of these minor matrices as a source of bio-compounds with application in foods can have a high economic importance, opening new markets and local supply chains for the extraction,

purification and formulation of new natural antimicrobials and antioxidants, while employing new raw materials that, currently, have no market or commercial value.

Given these considerations, the general aim of my PhD thesis, that is a part of the European project BioProMedFood, financed in the frame of PRIMA – Section 2 Programme, is to study sustainable approaches to increase the safety and quality of fresh and fermented meat products. With this purpose, three main strategies have been evaluated:

i) use of autochthonous strains, isolated from spontaneously fermented sausages collected in Mediterranean countries, as bio-protective cultures or functional starter cultures: with the aim to valorise the biodiversity and genetic bacterial heritage represented by traditional meat products of the Mediterranean area, LAB have been isolated, identified and characterised for their safety, technological and antimicrobial features. The most promising strains have been further tested for their bio-protective activities in fresh sausages and used as starter cultures in fermented sausages;

ii) use of natural compounds with antioxidant and antimicrobial potential: this strategy involves the extraction of bio-active compounds from low-cost sources or by-products from plants of the Mediterranean maquis. The phenolic extracts (PE) and the essential oils (EO) have been characterised and tested for their antimicrobial potential against some food-borne pathogens;

iii) use of fermentation of plant by-products to obtain plant extracts enriched in phenolic compounds: the use of peculiar LAB strains has been proposed for the fermentation of unconventional matrices to modify the availability of bio-active compounds.

To reach these key objectives, the work of this PhD thesis has been divided into several parts:

Part 1: Valorisation of spontaneously fermented sausages microbial biodiversity

Specific objectives:

1a) to characterise and investigate the microbial ecology in spontaneously fermented sausages, produced and collected from four different Mediterranean countries (Italy, Spain, Croatia, and Slovenia), using a combination of culture-dependent and metagenomic techniques. These products have been proposed as source of isolation of autochthonous LAB to exploit their bacterial biodiversity;

1b) to isolate, identify and assess the safety of autochthonous LAB, isolated from the spontaneously fermented sausages previously collected and characterised, with the aim to understand the ecology of these natural fermented meat products and to know which LAB species are the most abundant and persistent. The resulted strains have been studied regarding their

antibiotic-resistances and amino biogenic potential, since safety features are fundamental to further determine strains suitability to be used in foods as potential autochthonous starter cultures or bio-protective cultures;

1c) to characterise safe autochthonous strains as regard their antimicrobial potential, *i.e.* their ability to inhibit pathogen microorganisms *in vitro* and in meat model and to produce bacteriocins and their technological properties, *i.e.* growth kinetics at different salt concentrations and incubation temperatures.

Part 2: Use of new LAB cultures in meat products: evaluation of their bio-protective activities and technological performances

Specific objectives:

2a) to use two promising strains, endowed with bio-protective features, in challenge tests in fresh sausages against *Listeria monocytogenes* and *Salmonella enterica* serovar Enteritidis, to study their antimicrobial activity in food models in laboratory scale trials;

2b) to use two promising strains as starter cultures to produce Spanish fermented sausages (salchichón) in pilot plants. Fermentation performances, the product aroma profile and safety have been assessed and a challenge test was performed against *Listeria monocytogenes* to detect bio-protective activity of the proposed starters cultures.

Part 3: Study of the in vitro antimicrobial potential of bio-active compounds against spoilage microorganisms or food-borne pathogens

Specific objectives:

3a) to characterise the PE and EO obtained from *Rubus fruticosus* leaves and *Juniperus oxycedrus* needles, harvested in Croatia, and to study these under-exploited matrices as source of biologically active compounds;

3b) to study the antimicrobial potential of PE and EO against *Listeria monocytogenes* Scott A and *Enterococcus faecium* FC12. The bio-active substances have been employed at sub-lethal level to monitor and model the growth dynamics of the target pathogen or spoilage bacteria using predictive microbiology tools. In addition, to better evidence PE and EO effect on viability and physiological state of target microorganism cells, flow cytometric analyses were performed.

3d) to study the effects of the same PE and EO plant derivatives on the amino biogenic potential of *Ent. faecium* FC12. Its capability to produce tyramine has been studied in the presence of sub-lethal concentration of bio-active compound to assess their potential in reducing the accumulation of this toxic biogenic amine.

Part 4: Increasing of bio-active compounds availability in vegetal matrices fermented by LAB strains

Specific objectives:

4a) to study the capability of selected LAB strains to use non-conventional plant matrices, such as avocado leaves, as fermentation substrate;

4b) to evaluate the effect of fermentation process and microbial metabolism on the availability of phenolic compounds and to assess the antioxidant activity of fermented avocado leaf extracts.

Chapter 3:

Valorisation of spontaneously fermented sausages microbial biodiversity

3.1 Mediterranean spontaneously fermented sausages: spotlight on microbiological and quality features to exploit their bacterial biodiversity

3.1.1 Introduction

Fermented meat products represent an important industrial sector in Europe, particularly in the Mediterranean countries, and a valuable cultural heritage strongly linked to the identity of a population or specific production areas. In fact, a wide variety of sausages are produced using typical regional recipes and ancient processes (Leroy et al., 2015; Ojha et al., 2015). As highlighted by several authors, this geographical origin affected the microbiological features and technological characteristics of fermented foods, due to the specific manufacturing process conditions and different raw materials and formulations (Spitaels et al., 2014; Van Reckem et al., 2019).

For years, the meat industry was defined by massive technological, economic, social, and even nutritional transformations, among which innovation improving production and safety standards with reduction in waste, production time, energy and costs (Leroy et al., 2015). Among these approaches, the use of starter cultures was introduced to guide fermentation, enhancing product safety. On the other hand, this resulted in a loss of biodiversity and peculiar features of these traditional products (Cocconcelli and Fontana, 2010). Nevertheless, in the different areas of the Mediterranean countries, the presence of numerous local products still obtained through spontaneous fermentation can represent a relevant source of unexplored microbial biodiversity.

Spontaneous fermentations are characterised by presence of indigenous microorganisms that confer peculiar characteristics to the products in terms of both technological and organoleptic traits (Franciosa et al., 2018). The peculiar sausage flavour, aroma and texture formation are due to the interactions among raw materials, production process and the succession of different microorganisms, that are responsible for biochemical and physico-chemical reactions in which they cooperate within the meat matrix and its surface (Flores, 2018; Palavecino Prpich et al., 2021). In this context, LAB and coagulase-negative cocci (CNC) strains play a principal role in driving a controlled fermentation with a rapid environmental acidification and formation of desirable aroma and sensorial properties, respectively. Meanwhile, fungi and yeasts can exert important effects, preventing excessive dehydration and the oxidation of the lipid fraction due to oxygen (Franciosa et al., 2018). The study of traditional spontaneously fermented sausages characteristics and their microbiota can be a fundamental strategy to explore new technological and functional strains, that can be used into local productions in order to preserve authenticity and traditional features of these products.

The first part of the PhD project was focused on the characterisation and investigation of the microbial ecology in spontaneously fermented sausages, produced and collected from four different Mediterranean countries (Italy, Spain, Croatia, and Slovenia), using a combination of culture-dependent and metagenomic techniques. In addition, the safety (*i.e.* biogenic amines content) and physico-chemical features of the products, as well as their aroma profile, were evaluated.

3.1.2 Materials and methods

3.1.2.1 Spontaneously fermented sausages collection

A total of fifteen samples of artisanal fermented sausages, produced without the addition of starter cultures, were collected at the end of ripening from four different Mediterranean countries: two Italian salami, two traditional Slovenian smoked salami, seven traditional salchichón and chorizo produced in different locations from Andalusia (Spain) and three samples from Croatia (Table 3.1).

Table 3.1: Spontaneously fermented sausages collection.

Countries	Products	Samples name
Italy	Salame Fabriano_product 1 (Marche region)	IM1
	Salame Fabriano_product 2 (Marche region)	IM2
	Salame Alfianello (Lombardia region)	IAL
Slovenia	Traditional smoked salami with nitrates	SN
	Traditional smoked salami without nitrates	SWO
Spain	Salchichón Alhendín	ESA
	Salchichón Bérchules	ESB
	Salchichón Écija	ESE
	Salchichón Olvera	ESO
	Chorizo Bérchules	ECB
	Chorizo Écija	ECE
	Chorizo Olvera	ECO
Croatia	Traditional unsmoked salami	HNS
	Traditional smoked salami	HS
	Salami Zminjska Klobasica	HZK

3.1.2.2 Physico-chemical analysis

The pH of the 15 ripened samples was evaluated by using a pH-meter Basic 20 (Crison Instruments, Barcelona, Spain), while the water activity (a_w) was detected with an Aqualab CX3-TE (Labo-Scientifica, Parma, Italy). All measures were performed in triplicate.

Moreover, a FoodScan instrument (Foss, Hilleroed, Denmark) was used to carry out centesimal composition of the samples. This technique used near-infrared transmission (NIT), with

a wavelength between 850-1050 nm, for inhomogeneous samples. The absorbance data obtained were processed with a mathematical function and a calibration model to calculate the percentage (%) of fat, moisture, protein, collagen and salt.

3.1.2.3 Microbiological characterisation

The microbial composition of all fermented sausages at the end of ripening was evaluated through plate counting. The samples were prepared by removing aseptically the casing and a slice of approximately 10 g of the product was transferred into a stomacher bag, mixed with 90 ml of 0.9% (w/v) NaCl sterile solution and homogenised in a Lab Blender Stomacher (Seward Medical, London, UK) for 2 min. Afterwards, appropriate decimal dilutions were prepared and plated onto selective culture media for the detection of each microbial group of interest (Figure 3.1).

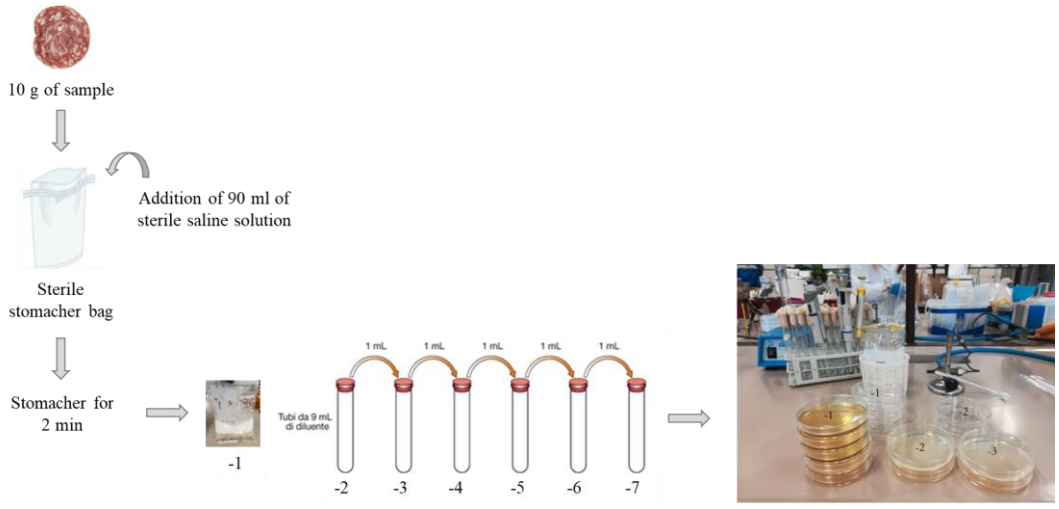


Figure 3.1: Microbiological sampling.

In particular, the following microbial populations were determined:

- Lactobacilli were enumerated by using Man-Rogosa-Sharpe (MRS) agar, added with 200 mg/l of cycloheximide (Sigma-Aldrich, St Louis, USA), an antibiotic that inhibits the yeasts and moulds growth. The obtained plates were incubated at 30° at 48 h;
- CNC were detected with Mannitol Salt Agar (MSA) after an incubation at 30°C for 48 h;
- Slanetz and Bartley medium (SL) was employed for enterococci enumeration. The plates were incubated at 44°C for 24 h;
- Yeasts and moulds, detected with Sabouraud Dextrose agar (SAB) added with 200 mg/l of chloramphenicol (Sigma-Aldrich) to inhibit the growth of bacteria, were counted after 72 h of incubation at 30°C;

- Violet Red Bile Glucose Agar was used for *Enterobacteriaceae*. The counts were collected after 24 h of incubation at 37°C.

The sampling was performed in triplicate for each product.

Moreover, the presence of the main food-borne pathogens was assessed. In particular, *E. coli* was enumerated with Violet Red Bile Agar medium, in which MUG (4-methylumbelliferyl-ßD-glucuronide) supplement was added to detect this microorganism if it is able to produce a fluorescent result. On the other hand, the presence/absence of *List. monocytogenes* and *Salmonella* were evaluated according to the methods EN ISO 11290-1 and EN ISO 6579-1, respectively (ISO, 2017a; ISO, 2017b). In the first case, 25 g of samples were added with 225 ml UVMI enrichment medium and incubated at 30°C for 24 h. Subsequently, 100 µl of sample were transferred into a tube containing 10 ml of UVMII medium and incubated again at 30°C for 24 h. At the same time, a further 10 ml of the sample was added to 4.5 ml of KOH solution and immediately streaked on Listeria Selective Agar Base, Oxford Formulation (LSO) plate (30ºC for 24 h). After incubation, 100 µm of UVMII sample were spread onto LSO plate and 1 ml was added to 4.5 ml of KOH solution and immediately streaked onto LSO plate. All the obtained plates were incubated at 30°C for 24 h. The presence/absence of *Listeria* was evaluated through the presence of black colonies, characterised by a crater in the central part, indicating the typical morphology of this microorganism. Regarding *Salmonella*, 25 g of sample were added to 225 ml of Buffered Peptone Water and incubated for 24 h at 30°C. Subsequently 100 µl of this solution were added into a tube containing 10 ml of Rappaport-Vassiliadis Enrichment Broth (RVS) medium, then incubated at 42°C for 24 h. Finally, to assess the presence of the target microorganism, the sample was streaked on two different specific media: XLD and Bismuth Sulphite Agar (BSA). The plates were incubated at 37°C and the results were observed after 24 h.

To perform these analyses, all media were provided by Oxoid (Basingstoke, UK).

3.1.2.4 DNA extraction, sequencing and bioinformatic analysis

Total genomic DNA was directly extracted from 200 mg of frozen products samples after a treatment with lysozyme at 37°C for 1 h, followed by mechanic lysis through TissueLyser II (Qiagen) with a frequency of 30 Hz for 5 min. The DNA was then purified using a Wizard genomic DNA purification kit (Promega, Mannheim, Germany) according to the manufacturer recommendations. The purified DNA resuspended in water was quantified using a Qubit 4 Fluorimeter (ThermoFisher Scientific, Waltham, MA, USA). After the normalisation of DNA concentration, the sequencing was carried out through the Illumina MiSeq platform, generating 300

bp pair-end sequencing reads. The library for Illumina sequencing was generated from V3-V4 variable regions of ribosomal 16S rRNA to characterise the bacterial population of the samples.

Bioinformatic elaboration data was performed through the analysis of FASTQ sequence files from Illumina reads by using DADA2 version 1.8 with the R 3.5.1 environment, which implements a new quality-aware model of Illumina amplicon errors without constructing OTUs (Callahan et al., 2016). The parameters applied were set in order to obtain trimLeft equal to 30 and truncLen option set to 270 and 200 for the forward and reverse FASTQ files, respectively. The comparison between the amplicon sequence variant (ASV) predicted from DADA2 against SILVA database (version 138 updated according to the reclassification of the genus *Bacillus* and *Lactobacillus*) was used for the taxonomic assignment. ASVs belonging to taxa classified as external sample or with low abundance setting (threshold of relative abundance equal to 0.5%) were not included in the composition analysis for microbial population (Davis et al., 2018). The assignment at the species level for the remaining ASV was achieved.

3.1.2.5 Biogenic amines determination

Biogenic amines (BA) quantification was determined for each product, after an extraction with trichloroacetic acid (TCA) 5%. Briefly, 10 g of sample were treated with 20 ml of TCA at 75°C for 30 min. After this incubation the solution was filtered into 50 ml flask. This step was repeated twice an at the end the extract acid solution was added until reach a final volume of 50 ml. Before the HPLC injection, the extracts were subjected to a dansyl-chloride derivatization (Sigma-Aldrich), according to Pasini et al. (2018).

An HPLC Agilent Technologies 1260 Infinity with the automatic injector (G1329B ALS 1260, loop of 20 µl), equipped with a UV detector (G1314F VWD 1260) set at 254 nm, was used to detect the presence of the principal BA (*i.e.* histidine, tyramine, putrescine, cadaverine and 2-phenylethylamine) (Figure 3.2).

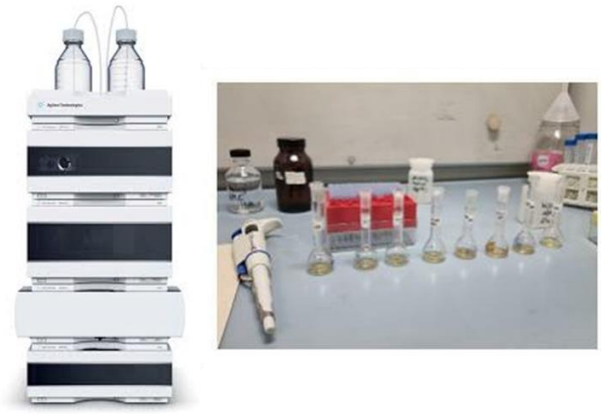


Figure 3.2: Biogenic amines quantification through HPLC injection.

For the chromatographic separation a C18 Waters Spherisorb ODS-2 (150 × 4.6 mm, 3 μm) column, left at 30°C for the entire analysis, was used with a gradient elution of acetonitrile and HPLC water (Sigma-Aldrich): 0-1 min acetonitrile/water 35:65, 1-6 min acetonitrile/water 55:45, 6-16 min acetonitrile/water 60:40, 16-24 min acetonitrile/water 90:10, 24-35 min acetonitrile/water 90:10, 35-40 min acetonitrile/water 35:65, 40-45 min acetonitrile/water 35:65, all at a flow rate 0.6 ml/min.

The BA amount was expressed as mg/kg with reference to a calibration curve obtained through aqueous dansyl-chloride-derivatized BA standards of concentrations ranging from 10 to 200 mg/l (Sigma-Aldrich). The detection limit for all these compounds was 3 mg/kg of the sample under the adopted conditions. All the analyses were performed in triplicate.

3.1.2.6 Aroma profile analysis

Gas-chromatography-mass spectrometry coupled with the solid-phase microextraction (GC-MS-SPME) technique was employed for the volatile organic compounds (VOCs) analysis of the products at the end of ripening (Montanari et al., 2016).

A total of 3 g of each product were placed into 10 ml sterilised vials, sealed by PTFE/silicon septa. The samples were stored at -20°C until the analysis. Before their characterisation, they were combined with a known amount of 4-methyl-2-pentanol (Sigma-Aldrich) as internal standard and heated for 10 min at 45°C. After this conditioning phase, a fused silica fibre covered by 75 mm carboxen polydimethyl siloxane (CAR/PDMS StableFlex) (Supelco, Steinheim, Germany) was introduced in the vial headspace for 40 min. Finally, the molecules were desorbed in the gas-chromatography instrument for 10 min.

An Agilent Hewlett Packard 7890 GC gas-chromatograph equipped with a MS detector 5975 MSD (Hewlett-Packard, Geneva, Switzerland) were used to peaks detection (Figure 3.3). Their chromatographic separation was performed by using a Varian (50 m × 320 μm × 1.2 μm) fused silica capillary column, in which the carrier gas (He) flowed at a rate of 1 ml/min.

Figure 3.3: Volatile organic compounds analysis through GC-MS-SPME technique.

Volatile peaks identification was carried out using Agilent Hewlett-Packard NIST 2011 mass spectral library (Gaithersburg, MD, USA) (NIST, 2011) and the data were expressed as the ratio between each molecules peak area and the peak area of internal standard. All the analyses were performed in triplicate.

3.1.2.7 Statistical analysis

Principal component analysis (PCA) and cluster analysis (LDA) were carried out using Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA).

3.1.3 Results and discussion

3.1.3.1 Geographical origin and manufacturing processes

The fifteen dry-fermented sausages, collected to perform their characterisation in this first part of the project, were produced according to traditional recipes, linked to their different Mediterranean countries of origin (Italy, Slovenia, Spain and Croatia), without the addition of starter cultures. The peculiar characteristics of these products are described in Table 3.2. In particular, the samples differed in their manufacturing process, varied in term of raw material, lean/fat meat ratio, type of casing, spices, addition of nitrates and nitrites, a possible smoking treatment, etc. In fact, the diameter of the products varied between 2.5 and 6 cm. Different spices have been used in the formulation: pepper, cloves, oregano, coriander, nutmeg, paprika, garlic, etc., depending on the sample considered and its origin. However, all products were obtained using only pork meat and fat, apart from two Slovenian samples (SN and SWO) that also contained 20% of beef meat. Moreover, the ripening conditions (temperature, time, etc.) differed between the samples, and only three samples were smoked, *i.e.* two Slovenian samples (SN and SWO) and a Croatian sample (HS sample).

Table 3.2: Main characteristics of spontaneously fermented sausages collected from four Mediterranean countries (Italy, Slovenia, Spain and Croatia) in terms of ingredients, type of casing, presence of preservatives and smoking phase.

Characteristics of the tested sausages	Italy			Slovenia		Spain							Croatia		
	IM1	**IM2**	**IAL**	**SN**	**SWO**	**ESA**	**ESB**	**ESE**	**ESO**	**ECB**	**ECE**	**ECO**	**HNS**	**HS**	**HZK**
Section															
Diameter (cm)	5	3.5	6	5	5	4	5.5	4.5	5.5	4.5	4	5	3.5	3.5	2.5
Type of lean meat	pork	pork	pork	pork/ bovine (3:1)	pork/ bovine (3:1)	pork	pork	pork	pork	pork	pork	pork	pork	pork	pork
Fat in the meat batter (%)	8-12	8-12	20-30	19	19	20-25	20-30	20-30	20-25	25-30	25-30	15-20	30-35	30-35	20
Fat characteristics	cubes	cubes	minced	cubes	cubes	minced	minced	minced	minced	minced	minced	minced	cubes	cubes	cubes
Spices	pepper, white wine	pepper, white wine	pepper, cinnamon, nutmeg, cloves	pepper, garlic	pepper, garlic	pepper, nutmeg	pepper, nutmeg	pepper, nutmeg	pepper, nutmeg	paprika, origan, nutmeg, coriander	paprika, origan, nutmeg, coriander	paprika, origan, nutmeg, coriander	pepper, garlic, mild paprika, hot paprika	pepper, garlic, mild paprika, hot paprika	pepper, garlic, wine
Nitrate/nitrite	no	no	yes	yes	no	yes	yes	yes	yes	no	no	no	no	no	no
Type of casing	natural	natural	natural (pork or bovine)	collagen	collagen	collagen	collagen	collagen	collagen	collagen	collagen	collagen	natural (pork intestine)	natural (pork intestine)	natural (pork intestine)
Smoking treatment	not smoked	not smoked	not smoked	smoked	smoked	not smoked	not smoked	not smoked	not smoked	not smoked	not smoked	not smoked	not smoked	smoked	not smoked

3.1.3.2 Physico-chemical characterisation

In Table 3.3 the physico-chemical parameters of the different fermented sausages at the end of ripening are reported. Wide differences in the final pH were observed, with values ranging from a minimum of 4.52 in the ECO sample to a maximum of 6.42 in IM1.

In general, Italian products presented the highest pH values, between 5.88 and 6.42, together with Croatian ones, ranging from 5.72 to 6.05. On the other hand, Slovenian sausages showed lower pH values (5.20 and 5.39). These values agree with those reported by Lešić et al. (2020) for Croatian and Slovenian sausages and with the data reported by Cardinali et al. (2018) for Italian Fabriano sausages. Slovenian products were probably more subjected to northern European production influences. In fact, northern products are generally dried sausages with a pH around or even below 5 and often undergo a smoking phase (that inhibits moulds), while Mediterranean products are usually long-ripened fermented sausages with pH values up to 6.2-6.4, given the possible growth of desirable moulds (Holck et al., 2015).

The Spanish samples were widely different in terms of pH, due to the heterogeneous traditional manufacturing process developed in the whole country. In fact, chorizo samples showed a very low pH, between 4.52 and 5.04, while the salchichón had a pH ranging from 5.13 to 5.83. Indeed, different information is reported in the literature for chorizo characteristics and, among them, the pH can vary (Prado et al., 2019; Rodríguez-González et al. 2020).

Great variability was also observed in the final a_w of the products. This parameter reached extremely low values, such as in IM2 (0.760), ESB (0.811), IM1 (0.824) and SN (0.823). On the other hand, HNS showed the highest value (0.928). In any case, the a_w values reflected the water content in the final products, that varied from 20.42% in IM2 to 39.04% in ESO. Finally, the salt content at the end of ripening ranged from 2.94 % (HNS) to 4.48 % (IM2).

These data reflected that all physico-chemical parameters considered were affected by the geographical origin and manufacturing processes (*e.g.* smoking).

Table 3.3: Physico-chemical parameters and composition of the fifteen spontaneously fermented sausages at the end of ripening. Standard deviation is also reported.

Physico-chemical parameters	Italy		Slovenia			Spain							Croatia		
	IM1	**IM2**	**IAL**	**SN**	**SWO**	**ESA**	**ESB**	**ESE**	**ESO**	**ECB**	**ECE**	**ECO**	**HNS**	**HS**	**HZK**
pH	6.42 ± 0.02	6.09 ± 0.01	5.88 ± 0.03	5.20 ± 0.02	5.39 ± 0.01	5.83 ± 0.03	5.63 ± 0.04	5.80 ± 0.02	5.13 ± 0.03	4.77 ± 0.02	5.04 ± 0.01	4.52 ± 0.03	5.81 ± 0.05	5.72 ± 0.04	6.05 ± 0.03
a_w	0.824 ± 0.003	0.760 ± 0.002	0.879 ± 0.002	0.823 ± 0.002	0.832 ± 0.003	0.917 ± 0.002	0.811 ± 0.002	0.848 ± 0.003	0.911 ± 0.004	0.895 ± 0.001	0.870 ± 0.003	0.908 ± 0.001	0.928 ± 0.001	0.903 ± 0.003	0.890 ± 0.001
Humidity (%)	26.15 ± 0.35	20.42 ± 0.38	30.11 ± 0.40	25.05 ± 0.27	26.07 ± 0.38	38.54 ± 0.19	25.45 ± 0.22	27.60 ± 0.18	39.04 ± 0.44	31.12 ± 0.30	30.25 ± 0.41	38.54 ± 0.29	28.75 ± 0.33	25.11 ± 0.38	32.69 ± 0.23
Fat (%)	34.13 ± 0.31	35.79 ± 0.33	34.18 ± 0.26	40.65 ± 0.17	41.33 ± 0.41	29.75 ± 0.28	42.15 ± 0.30	36.05 ± 0.35	29.42 ± 0.21	43.75 ± 0.25	40.38 ± 0.29	29.71 ± 0.37	50.37 ± 0.40	47.21 ± 0.46	30.21 ± 0.26
Proteins (%)	34.32 ± 0.21	37.73 ± 0.35	29.27 ± 0.17	27.61 ± 0.60	26.15 ± 0.44	26.47 ± 0.29	26.83 ± 0.33	28.48 ± 0.24	25.20 ± 0.48	18.44 ± 0.41	22.53 ± 0.26	23.71 ± 0.35	15.14 ± 0.40	20.08 ± 0.29	30.29 ± 0.34
Collagen (%)	1.12 ± 0.05	1.62 ± 0.06	2.61 ± 0.07	3.25 ± 0.03	3.23 ± 0.09	1.02 ± 0.10	1.28 ± 0.04	3.72 ± 0.06	2.10 ± 0.09	3.34 ± 0.08	2.69 ± 0.05	3.61 ± 0.04	2.81 ± 0.11	3.84 ± 0.07	2.08 ± 0.10
Salt (%)	4.31 ± 0.10	4.48 ± 0.05	3.84 ± 0.09	3.44 ± 0.07	3.24 ± 0.11	4.26 ± 0.02	4.24 ± 0.08	4.10 ± 0.06	4.28 ± 0.12	3.31 ± 0.05	4.12 ± 0.04	4.42 ± 0.08	2.94 ± 0.09	3.78 ± 0.05	4.69 ± 0.07

3.1.3.3 Microbiological analysis of the different spontaneously fermented sausages

Microbial counts were performed to enumerate in the final product LAB, CNC, enterococci, enterobacteria (including *E. coli*) and yeasts (Table 3.4).

Among the researched pathogens, *E. coli* were always below the detection limit (<1 log cfu/g). Moreover, no positive samples for the presence of *List. monocytogenes* and *Salmonella* were found in final products.

Table 3.4: Concentrations (log cfu/g) of the main microbial groups in the fifteen sausages at the end of ripening.

Microbial groups	Italy			Slovenia			Spain						Croatia		
	IM1	**IM2**	**IAL**	**SN**	**SWO**	**ESA**	**ESB**	**ESE**	**ESO**	**ECB**	**ECE**	**ECO**	**HNS**	**HS**	**HZK**
Lactobacilli	7.07 ± 0.48	8.52 ± 0.11	8.26 ± 0.16	6.57 ± 0.02	7.28 ± 0.28	7.85 ± 0.75	6.96 ± 0.21	6.32 ± 0.30	7.78 ± 0.14	4.41 ± 0.72	5.88 ± 1.02	7.73 ± 0.27	8.67 ± 0.11	8.43 ± 0.09	8.54 ± 0.03
CNC	7.12 ± 0.09	7.13 ± 0.12	5.22 ± 1.05	<1*	1.44 ± 2.03	3.65 ± 0.22	3.05 ± 4.31	4.54 ± 0.16	5.40 ± 0.12	<1	<1	<1	5.34 ± 0.82	5.09 ± 0.01	7.24 ± 0.32
Enterococci	<1	<1	<1	<1	<1	<1	2.37 ± 0.10	2.19 ± 0.53	2.05 ± 0.38	<1	1.19 ± 0.35	<1	3.74 ± 0.12	3.18 ± 0.74	4.80 ± 0.16
Yeasts and moulds	5.44 ± 0.05	5.01 ± 0.26	3.46 ± 0.49	0.95 ± 1.35	3.26 ± 0.37	5.34 ± 0.25	3.41 ± 0.80	4.10 ± 0.58	4.59 ± 0.59	3.10 ± 0.27	2.60 ± 0.25	3.70 ± 0.08	2.27 ± 0.31	3.85 ± 0.31	4.15 ± 0.10
Enterobacteria	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	3.55 ± 0.50	2.71 ± 0.82	5.12 ± 0.49

*<1: under the detection limit

The dominant microbial population was generally represented by LAB, whose counts in most of samples ranged from about 7.0 (IM1 and ESB) to 8.7 (HNS) log cfu/g. The highest LAB counts were found in samples collected from Croatia and Italy. Conversely, the lowest numbers of this microbial group were associated with sausages from Spain, in particular ECB (4.4 log cfu/g) and ECE (5.9 log cfu/g). It is well known that LAB are the principal microorganisms that dominate the microbiota of traditionally fermented sausages, with loads of 7-8 log cfu/g in the final products (Leroy et al., 2013).

The counts of CNC were higher in Italian Fabriano salami (IM1 and IM2), with a concentration of 7.1 log cfu/g, and in the Croatian HZK (7.2 log cfu/g). This microbial group was below the detection limit in the samples SN, ECB, ECE and ECO, while in SWO, its concentration was very low (1.4 log cfu/g). The presence of CNC was strongly influenced by pH, since they seem to be inhibited by lower pH.

Enterococci were not detected (<1 log cfu/g) in Italian and Slovenian samples and in three Spanish samples (ESA, ECB, ECO). On the contrary, the highest counts of this microbial group were found in Croatian samples (3.2-4.8 log cfu/g).

Yeasts ranged from 2.3 to 5.4 log cfu/g, except for SN sample, characterised by a very low load. This microbial population can have an important role in sausage-ripening, contributing to the formation of the aroma and to the evolution of product sensory features (Flores et al., 2015).

Finally, *Enterobacteriaceae* were present in detectable amounts only in Croatian products, with values ranging between 2.7 (HS) and 5.1 (HZK) log cfu/g. In all other samples, this population was below the detection limit. High levels of *Enterobacteriaceae* can indicate the low microbiological quality of the product, and high concentrations (>4 log cfu/g) of this population were detected in several ripened traditional products (Roccato et al., 2017).

3.1.3.4 Metagenomic analysis

A more detailed picture of the bacterial composition of spontaneously fermented sausage was obtained through amplicon sequencing and metagenomic analysis.

Only species and genera that reached a concentration higher than 0.5% of amplicon sequence variants (ASVs) in at least one of the samples were considered. A total of more than 500 ASVs were detected, indicating very high biodiversity in the composition of the microbiota of the Mediterranean products taken into consideration. In Figure 3.4 the relative abundance of ASVs attributed to species is represented, while their percentages are reported in Table 3.5.

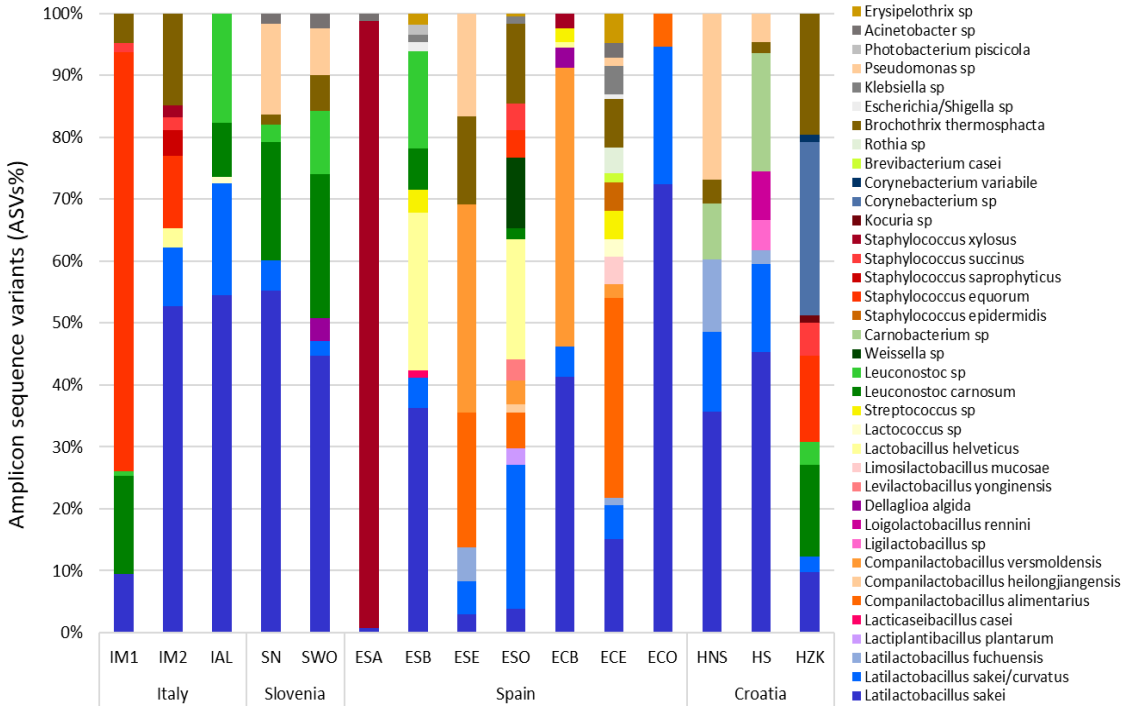


Figure 3.4: Microbial communities composition of the fifteen spontaneously fermented sausages analysed, expressed as relative abundance of amplicon sequence variants (ASVs).

Table 3.5: Relative abundance (%) of amplicon sequence variants (ASVs) in the fifteen fermented sausage samples investigated by metagenomic analysis. Only species and genera which reached a concentration higher than 0.5% in at least one of the samples are reported.

Species and genera detected	Italy		Slovenia			Spain							Croatia		
	IM1	**IM2**	**IAL**	**SN**	**SWO**	**ESA**	**ESB**	**ESE**	**ESO**	**ECB**	**ECE**	**ECO**	**HNS**	**HS**	**HZK**
Latilactobacillus sakei	9.51	52.71	54.54	55.24	44.75	0.71	36.32	2.89	3.88	41.24	15.12	72.43	35.68	45.36	9.72
Latilactobacillus sakei/curvatus	-*	9.55	17.99	4.94	2.34	-	4.85	10.87	23.16	4.95	6.67	22.25	24.63	16.44	2.57
Latilactobacillus fuchuensis	-	-	-	-	-	-	-	5.54	-	-	1.15	-	11.70	2.33	-
Lactiplantibacillus plantarum	-	-	-	-	-	-	-	-	2.64	-	-	-	-	-	-
Lacticaseibacillus casei	-	-	-	-	-	-	1.16	-	-	-	-	-	-	-	-
Companilactobacillus alimentarius	-	-	-	-	-	-	-	21.75	5.81	-	32.32	5.32	-	-	-
Companilactobacillus heilongjiangensis	-	-	-	-	-	-	-	-	1.32	-	-	-	-	-	-
Companilactobacillus versmoldensis	-	-	-	-	-	-	-	33.58	3.96	44.98	2.20	-	-	-	-
Ligilactobacillus sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	4.80	-
Loigolactobacillus rennini	-	-	-	-	-	-	-	-	-	-	-	-	-	7.94	-
Dellaglioa algida	-	-	-	-	3.66	-	-	-	-	3.32	-	-	-	-	-
Levilactobacillus yonginensis	-	-	-	-	-	-	-	-	3.36	-	-	-	-	-	-
Limosilactobacillus mucosae	-	-	-	-	-	-	-	-	-	-	4.44	-	-	-	-
Lactobacillus helveticus	-	3.07	-	-	-	-	25.54	-	19.45	0.94	-	-	-	-	-
Lactococcus sp.	-	-	1.12	-	-	-	-	-	-	-	2.72	-	-	-	-
Streptococcus sp.	-	-	-	-	-	-	3.68	-	-	2.09	4.61	-	-	-	-
Leuconostoc carnosum	15.84	-	8.75	19.06	23.26	-	6.70	-	1.70	0.09	-	-	-	-	14.80
Leuconostoc sp.	0.71	-	17.60	2.74	10.20	-	15.63	-	-	-	-	-	-	-	3.65
Weissella sp.	-	-	-	-	-	-	-	-	11.40	-	-	-	-	-	-
Carnobacterium sp.	-	-	-	-	-	-	-	-	-	-	-	-	9.00	19.11	-
Staphylococcus epidermidis	-	-	-	-	-	-	-	-	-	-	4.58	-	-	-	-
Staphylococcus equorum	67.74	11.65	-	-	-	-	-	-	4.47	-	-	-	-	-	14.04
Staphylococcus saprophyticus	-	4.20	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus succinus	1.47	2.09	-	-	-	-	-	-	4.35	-	-	-	-	-	5.27
Staphylococcus xylosus	-	1.81	-	-	-	98.13	-	-	-	2.38	-	-	-	-	-
Kocuria sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.25
Corynebacterium sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	27.91
Corynebacterium variabile	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.21

Brevibacterium casei	-	-	-	-	-	-	-	-	-	-	1.59	-	-	-	-
Rothia sp.	-	-	-	-	-	-	-	-	-	-	4.10	-	-	-	-
Brochothrix thermosphacta	4.73	14.91	-	1.73	5.81	-	-	14.23	12.77	-	7.90	-	3.91	1.65	19.58
Escherichia/Shigella sp.	-	-	-	-	-	-	1.45	-	-	-	0.68	-	-	-	-
Klebsiella sp.	-	-	-	-	-	-	1.21	-	1.20	-	4.56	-	-	-	-
Pseudomonas sp.	-	-	-	14.64	7.57	-	-	16.68	-	-	1.42	-	26.79	4.70	-
Photobacterium piscicola	-	-	-	-	-	-	1.67	-	-	-	-	-	-	-	-
Acinetobacter sp.	-	-	-	1.64	2.42	1.16	-	-	-	-	2.26	-	-	-	-
Erysipelothrix sp.	-	-	-	-	-	-	1.80	-	0.52	-	4.84	-	-	-	-

*: not detected

Beyond a large number of microbial species, their relative composition was also very variable among the products, even for those collected in the same geographical area. While in some samples most of the ASV were attributed to a single group/species, some fermented sausages were characterised by higher biodiversity, with an important diversification in the microbiota composition, as in the case of HZK and ESO.

Among LAB, several genus and species were present. Members of the genus *Latilactobacillus* were found in all the sausages and, in this context, *Lat. sakei* was the dominant species detected, in particular in IM2, IAL and SN samples (>50% of ASV). *Lat. sakei* and, to a lesser extent, *Lat. curvatus* have been reported as the prevailing LAB species in fermented meat products originating from France, Italy and Spain (Cocolin et al., 2011; Van Reckem et al., 2019). In fact, LAB species diversity of fermented sausages is known to be limited, being *Lat. sakei* predominant during the ripening process due to its excellent adaptation, competitiveness and assertiveness in the meat matrix (Aquilanti et al., 2016; Janßen et al., 2018; Montanari et al., 2018a). This predominance over other LAB can be attributed to its salt-tolerant and psychotropic nature, as well as its specialisation in metabolic pathways in the meat environment, including the arginine deiminase pathway and the utilization of nucleosides (Rimaux et al., 2011; Montanari et al., 2018b).

Other lactobacilli were sporadically detected in low amounts, such as *Lacticaseibacillus casei* in ESB, *Lactiplantibacillus plantarum* in ESO, *Ligilactobacillus* sp. and *Loigolactobacillus rennini* in HS and *Dellaglioa algida* in SWO. High levels of members of the genus *Companilactobacillus* (*Comp. alimentarius, Comp. heilongjiangensis* and *Comp. versmoldensis*) were present in many Spanish samples, in particular ESE and ECB, in which they represented 55.3% and 45.0% of the total ASV, respectively. The abundance of *Companilactobacillus* found only in Spanish products is a regional peculiarity already reported in the literature (Fontán et al., 2007a,b).

Heterofermentative lactobacilli, *Levilactobacillus yonginensis* and *Limosilactobacillus mucosae*, were present only in ESO and ECE, respectively. In addition, ASV belonging to some dairy LAB (*i.e. Lactobacillus helveticus, Streptococcus thermophilus* and *Lactococcus* sp.) were found in some Spanish products. These species can be related to the use of powdered milk or other dairy derivatives during manufacturing. The heterofermentative cocci were represented by *Leuconostoc* sp. (17.6% in IAL and 15.6% in SWO), *Leuconostoc carnosum*, present in relevant percentages in IM1 (15.8%), SN (19.1%), SWO (23.3%) and HZK (14.8%), and *Weissella* sp. found only in ESO.

The presence of staphylococci was detected only in some products. They belonged to the species *Staphylococcus epidermidis*, *Staphylococcus equorum*, *Staphylococcus saprophyticus*, *Staphylococcus succinus* and *Staph. xylosus* and were dominant in IM1 (67.7% of *Staph. equorum* ASVs and 1.5% of *Staph. succinus*) and ESA (98.1% of ASV belonging to *Staph. xylosus*). Among CNC, *Staph. xylosus* is the prevalent species associated with fermented sausages, even if a greater variability can be found, that results in the presence also of *Staph. epidermidis*, *Staph. equorum* and *Staph. saprophyticus*. Their persistence is assured by the use of alternative energy sources, such as arginine and nucleoside. In addition, these microorganisms exert several important technological roles in fermented sausage production, such as flavour and colour formation (Sánchez Mainar et al., 2017). Staphylococci were detected as subdominant fractions in several fermented meat products, but the very low pH that characterised some samples, *i.e.* Spanish products, could hinder their growth. The results therefore underlined the sensitivity of this genus to the strong initial acidification in these products, which was not followed by a significant pH increase (scarcity of moulds, etc.).

Carnobacterium sp. was detected only in some Croatian samples (HS and HNS), although with a relevant percentage (19.11% and 9%, respectively). This genus has been associated with meat spoilage phenomena, as reported by Casaburi et al. (2011). Among *Actinobacteria*, *Kocuria* sp. and *Corynebacterium* sp. were present only in HZK, while *Brevibacterium casei* and *Rothia* sp. were present in ECE.

The meat spoiler *Brochothrix thermosphacta* was detected in several sausages and its concentration was particularly relevant in HZK (19.58%), IM2 (14.91%) and ESE (14.23%). This species has been reported to be part of the Fabriano-like sausage microbiota by Cardinali et al. (2018) and it is commonly associated with spoiled meat-based products (Raimondi et al., 2018). Among Gram-negative bacteria, *Pseudomonas* sp. appeared at a high concentration in Slovenian samples and Spanish ESE product (16.7% of ASVs), but the higher presence was found especially in HNS, in which represented 26.8% of ASVs. Finally, *Enterobacteria* (*Klebsiella* sp. and *Escherichia*/*Shigella* group) were found in ESB, ESO and ECE.

To better evidence the differences in the ASVs composition, a cluster analysis was carried out and the results are reported in Figure 3.5. Four different clusters were obtained. The first contained only the Italian sausage IM1. In the second cluster three Spanish sausages (ESE, ECE and ESO) and the Croatian sample HZK were grouped. The third cluster was represented only by ESA. The fourth cluster included nine samples, and in particular the Spanish sausages ECB, ECO and ESB, the Croatian HNS and HS, the Slovenian sausages, and the Italian samples IM2 and IAL.

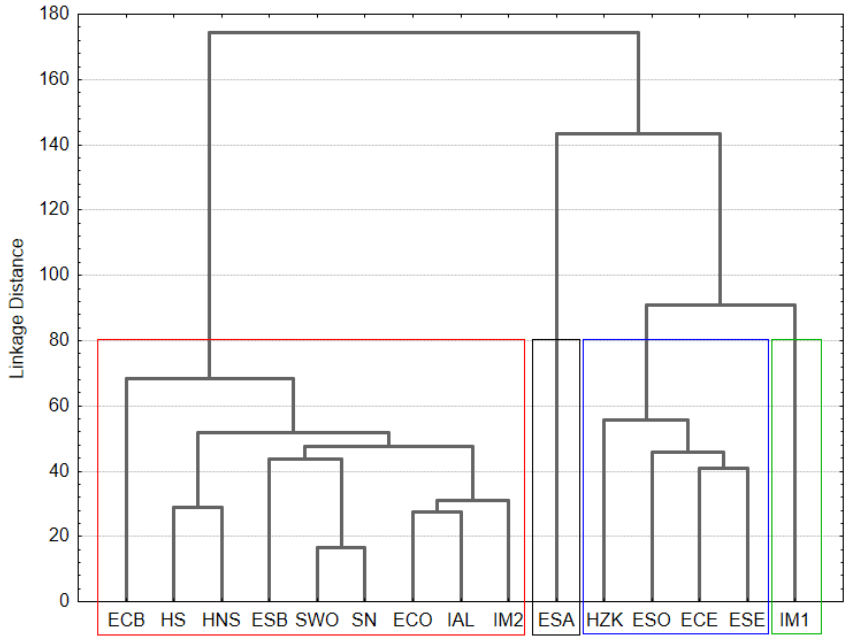
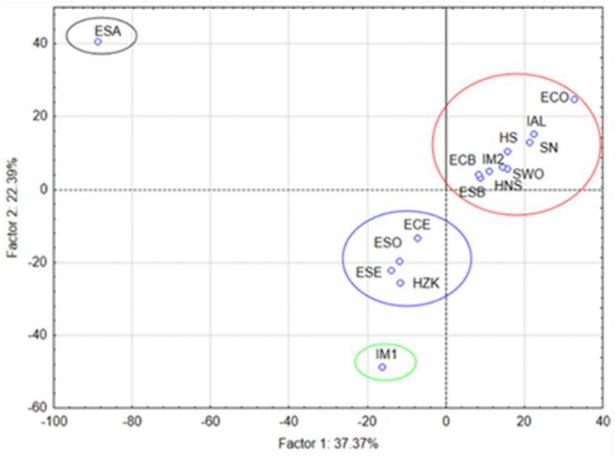


Figure 3.5: Dendrogram in which the analysed samples are grouped into 4 different cluster based on their ASVs composition.

To highlight the microorganisms responsible for the clustering of sausages, a PCA was applied whose first two components explained 59.76% of the variability (Figure 3.6).



Metagenomic variables	Factor 1	Factor 2
Latilactobacillus sakei	18.45	12.37
Lactobacillus sakei /curvatus	3.76	1.44
Companilactobacillus alimentarius	-1.09	-2.84
Companilactobacillus versmoldensis	-	-2.11
Leuconostoc carnosum	1.35	-2.03
Leuconostoc sp.	1.68	-
Staphylococcus equorum	-2.81	-11.76
Staphylococcus xylosus	-21.46	12.85
Corynebacterium sp.	-	-2.28
Brochothrix thermosphacta	-	-3.89
Pseudomonas sp.	1.35	-

Figure 3.6: Principal component analysis (PCA) that explain the metagenomic analysis results. The factor coordinates of the most relevant metagenomic variables (characterised by an absolute values higher than 1) for the first two factors are also reported, which represent the projection of the metagenomic variables on the factor-plane.

The grouping of the sausages reflected the results reported by the dendrogram (Figure 3.5). *Lat. sakei* was the main species responsible for the description of the largest group located in the first quadrant, while *Staph. xylosus* determined the collocation of ESA in the left part of the second quadrant. The position of IM1 depends on the variable *Staph. equorum*, while the remaining group collocation is mainly influenced by the presence of *Companilactobacillus*, especially for the

Spanish samples, *Corynebacterium* for the Croatian sausage and *Brochothrix thermosphacta* for all the samples grouped in this cluster.

3.1.3.5 Determination of biogenic amines content

BA concentrations in the analysed fermented sausages at the end of ripening are reported in Table 3.6.

Tyramine was detected in all samples, with concentrations ranging from 47.7 mg/kg (IM2) to 366.8 mg/kg (HNS). The mean content of this biogenic amine was 165.5 mg/kg, with a standard deviation of 88.8 mg/kg, indicating a fair variability among the fifteen samples. These amounts are similar to those reported by EFSA (2011), which indicated tyramine mean concentration of 136 mg/kg, with the 95^{th} percentile of 397 mg/kg in 400 European fermented sausages samples. In general, the Italian products showed lower tyramine concentrations, while the highest amounts were found in the Croatian samples HNS and HS.

Histamine was found only in two Spanish samples (ESB and ECE) at concentrations of 195.8 mg/kg and 174.7 mg/kg, respectively. This BA is considered the most dangerous for human health as it can cause various adverse effects known as “histamine poisoning” (Landete et al., 2008). Nevertheless, the quantities found in these samples, although not very high, are significant, especially when compared with the maximum amounts allowed in some fish products. These latter are the only ones regulated for histamine presence and generally admit a maximum quantity of 100 mg/kg in fresh fish and of 200 mg/kg for processed products (European Commission, 2005).

The presence of putrescine was more variable: in four samples this BA was not detected, while two Croatian samples (HNS and HS) showed the highest amounts, that was about 300 mg/kg. In the same samples, higher quantities of cadaverine were also observed.

Both Gram-negative and Gram-positive bacteria have been described as BA producers, with wide variability in amino biogenetic potential between different strains of the same species. Spoilage microorganisms, such as enterobacteria and pseudomonads, can be strong histamine, cadaverine and putrescine accumulators. BA produced by these microbial populations can also be found in fermented sausages (Gardini et al., 2016). On the other hand, decarboxylase activity has been found in Gram-positive strains, also belonging to the genus *Staphylococcus* and LAB. Although *Lat. sakei* is usually known for its inability to produce BA, many other LAB species are characterised by the presence of strains with decarboxylase activity. *Lat. curvatus*, for example, can accumulate both tyramine and histamine as well as *Comp. alimentarius*. The ability to produce BA has been found in other genera found in this investigation, such as *Leuconostoc* sp., *Weissella* sp. and *Carnobacterium* (Barbieri et al., 2019).

Table 3.6: Concentrations (mg/kg) of the main biogenic amines detected in the fifteen samples at the end of ripening.

Biogenic amines	Italy			Slovenia			Spain						Croatia		
	IM1	IM2	IAL	SN	SWO	ESA	ESB	ESE	ESO	ECB	ECE	ECO	HNS	HS	HZK
Histamine	-*	-	-	-	-	-	195.79 ± 27.29	-	-	-	170.74 ± 28.54	-	-	-	-
Tyramine	73.87 ± 21.83	47.65 ± 12.93	78.66 ± 1.31	209.38 ± 0.71	180.52 ± 10.34	199.24 ± 30.75	171.35 ± 37.52	149.92 ± 31.19	67.96 ± 14.34	146.06 ± 48.04	173.72 ± 39.46	202.50 ± 8.04	366.78 ± 38.31	312.93 ± 26.46	105.31 ± 29.18
Putrescine	-	-	115.67 ± 3.78	59.03 ± 2.70	67.58 ± 3.64	-	108.07 ± 15.41	42.79 ± 8.54	110.54 ± 4.26	99.28 ± 13.57	79.30 ± 10.81	155.95 ± 15.52	256.59 ± 8.92	359.59 ± 80.64	-
Cadaverine	-	-	-	83.38 ± 2.61	100.87 ± 0.44	67.94 ± 1.91	136.90 ± 20.01	-	-	-	-	-	436.03 ± 29.87	252.52 ± 30.31	-
TOTAL	73.87	47.65	194.33	268.41	348.98	267.18	612.10	192.72	178.50	245.34	423.76	358.46	1059.40	925.04	105.31

*: under the detection level (3-5 mg/kg)

3.1.3.6 Fermented sausages aroma profile

The aroma profile of the fifteen spontaneously fermented sausages at the end of the ripening was studied. The analysis of the volatile organic compounds (VOCs) allowed a clear differentiation of the samples, reflecting the different formulations, production and ripening conditions traditionally adopted in the Mediterranean geographical areas. Indeed, it has been reported that these differences can influence product aroma, being dry fermented sausage flavour affected by many processing factors, *i.e.* raw materials, spices, microbiota composition, smoking, etc. (Laranjo et al., 2017).

Within this wide variability, some common characteristics can be found by grouping the identified molecules in homogeneous chemical groups: ketones, aldehydes, alcohols, acids, and esters, as well as molecules deriving from spices and smoking (Table 3.7). The unidentified compounds accounted for less than 1% of the total peak area in each sample.

Concerning the molecules associated with the spices included in the meat batter formulation, they belonged to terpenes and terpenoids, phenylpropenes and compounds deriving from garlic (dimethyl disulfide, diallyl sulfide, etc.). These latter compounds were particularly present in Slovenian samples, Spanish chorizo and Croatian products, except for the HNS sample. Among terpenes, D-limonene certainly was the prevalent molecule in products in which pepper was added. Many other terpenes deriving from this spice, such as myrcene, linalool, copaene, carene, p-cymene, etc., were detected in these products, while they were absent in chorizo sausages, where oregano was used (Menon and Padmakumari, 2005). On the other hand, eugenol, safrole and methyl-eugenol are associated with the use of spices such as nutmeg, cinnamon and cloves (Batiha et al., 2020). The VOCs derived from smoke include furans and phenols, already reported for smoked products, and their presence was higher in the Slovenian fermented sausages (SN and SWO) and HS, characterised by a smoking phase during their production (Sikorski and Sinkiewicz, 2015). Furthermore, some of these VOCs were detected also in the Spanish chorizo samples, due to the traditional use of smoked paprika (Pereira et al., 2019).

Table 3.7: Volatile organic compounds (VOCs) detected by SPME-GC-MS in the samples, expressed as ratio between peak area of each molecule and peak area of the internal standard (4-methyl-2-pentanol). Compounds derived from spices or garlic and from smoking treatment are also reported. The standard deviation was always below 5%.

VOCs	Italy			Slovenia					Spain				Croatia		
	IM1	**IM2**	**IAL**	**SN§**	**SWO§**	**ESA**	**ESB**	**ESE**	**ESO**	**ECB†**	**ECE†**	**ECO†**	**HNS**	**HS§**	**HZK**
Acetone	5.85	3.00	2.61	0.87	1.31	-*	3.70	3.20	1.79	2.26	13.48	4.85	1.86	0.66	1.38
2-butanone	0.55	1.08	1.13	-	0.44	-	1.42	0.86	0.97	47.36	2.70	2.51	94.05	3.50	1.30
2,3-butanedione	1.87	1.54	0.23	0.41	0.48	0.18	0.50	2.44	0.52	5.93	3.68	1.32	2.14	3.42	2.03
2-pentanone	0.73	0.52	0.51	-	-	-	-	1.45	-	-	1.53	-	-	-	-
Methyl Isobutyl Ketone	0.89	0.81	0.70	0.62	0.50	0.61	0.64	0.52	2.87	0.79	2.46	3.00	2.97	3.94	3.90
4-methyl-3-penten-2-one	3.61	3.42	3.51	2.87	2.87	2.03	2.98	2.96	7.43	1.84	6.52	7.72	7.48	5.96	10.08
2,6-dimethyl-4-heptanone	-	-	-	-	-	-	-	-	-	0.65	5.39	6.87	3.20	3.42	-
2-heptanone	1.71	4.15	1.99	1.39	1.02	-	4.94	2.44	3.14	-	4.32	1.70	2.76	-	-
3-octanone	1.10	0.87	0.72	0.41	0.28	-	0.48	0.34	2.88	-	2.65	3.23	1.55	-	1.76
2-octanone	1.13	1.44	0.72	0.86	1.64	1.01	0.97	1.08	1.60	1.32	2.37	2.57	2.03	1.06	0.96
3-hydroxy-2-butanone	3.72	2.85	0.71	10.72	7.98	-	1.27	9.37	1.57	4.19	-	10.74	9.03	-	11.78
2,5-octanedione	0.39	4.64	0.72	1.34	0.78	-	-	-	2.84	-	-	-	14.68	-	-
2-nonanone	1.06	4.40	0.81	-	-	-	5.74	0.30	1.58	-	-	0.70	1.49	-	-
2-undecanone	0.84	0.65	-	0.78	0.71	-	3.06	0.40	-	-	-	-	1.47	2.88	-
Ketones	**23.46**	**29.36**	**14.35**	**20.26**	**18.02**	**3.83**	**25.70**	**25.37**	**27.18**	**64.33**	**45.09**	**45.21**	**144.71**	**24.84**	**33.19**
3-methyl-butanal	1.03	0.38	0.73	-	0.29	-	1.65	1.74	-	-	-	-	1.20	-	-
Pentanal	0.44	2.01	0.69	0.70	0.90	0.21	0.95	0.74	4.22	-	-	0.65	6.56	-	-
Hexanal	4.90	38.66	6.59	3.38	3.51	1.09	1.56	5.77	48.94	0.81	3.29	3.44	110.21	-	2.92
Heptanal	-	-	-	-	-	-	-	-	-	-	-	-	6.53	-	-
Octanal	-	-	-	-	-	-	-	-	-	-	2.46	-	5.10	1.47	-
2-heptenal	-	-	-	-	-	-	-	-	-	-	-	-	51.97	-	-
Nonanal	4.01	6.33	4.60	6.75	3.97	3.92	4.37	3.45	6.11	1.03	11.14	8.97	14.11	7.47	7.85
Decanal	1.72	2.59	1.57	-	-	1.27	2.63	0.91	2.01	0.78	-	-	5.16	-	-
Benzaldehyde	3.64	3.99	1.58	2.06	3.18	0.56	3.70	1.37	1.14	3.80	8.98	25.37	38.39	6.26	2.24
Benzeneacetaldehyde	5.47	4.03	14.61	5.30	13.70	6.27	6.28	5.66	4.85	1.30	4.82	2.49	30.31	127.99	23.42
Hexadecanal	1.31	2.93	2.63	1.40	1.61	1.10	1.39	1.03	1.30	3.49	5.58	3.26	-	-	-
Aldehydes	**22.52**	**60.92**	**33.00**	**19.59**	**27.17**	**14.42**	**22.54**	**20.67**	**68.57**	**11.21**	**36.27**	**44.18**	**269.54**	**143.19**	**36.43**
Ethyl alcohol	15.46	6.17	20.31	24.81	18.94	270.32	101.21	20.17	173.69	41.81	29.99	18.04	71.23	118.85	52.12
2-butanol	-	-	-	-	-	-	-	-	-	0.49	-	-	4.72	3.87	0.83
1-propanol	-	-	-	-	-	-	-	-	-	9.77	4.51	-	6.49	14.93	-
2-propen-1-ol	-	-	-	0.67	0.46	-	-	-	-	0.33	1.42	1.29	3.22	-	0.41
Isoamyl alcohol	0.66	0.44	0.35	0.49	0.40	6.19	2.10	3.45	2.66	0.51	2.13	1.03	3.72	-	1.73
1-pentanol	0.43	1.04	0.87	0.79	0.41	-	2.07	0.91	1.21	-	-	0.67	5.46	-	-
1-hexanol	2.08	3.88	2.00	1.69	2.26	2.68	1.89	5.01	8.71	6.98	5.48	3.96	-	3.00	-
1-octen-3-ol	2.85	5.01	1.30	0.62	1.08	-	0.49	1.41	2.81	0.44	2.70	1.19	19.92	1.52	0.84
1-octanol	0.69	0.88	0.67	0.81	0.62	0.52	0.55	0.94	0.61	0.41	1.36	0.93	3.09	1.26	1.03

Benzyl Alcohol	0.94	-	0.37	0.85	0.93	0.82	1.06	0.91	1.08	3.43	2.12	4.85	43.88	11.04	-
Phenylethyl Alcohol	1.16	1.17	0.59	2.26	3.40	7.85	1.62	3.01	1.70	-	4.11	2.84	5.05	28.23	1.45
Alcohols	**24.27**	**18.59**	**26.46**	**32.99**	**28.50**	**288.37**	**111.00**	**35.81**	**192.46**	**64.17**	**53.81**	**34.81**	**166.79**	**182.70**	**58.40**
Acetic acid	11.28	25.95	27.28	95.75	79.25	11.72	87.10	14.38	65.64	203.95	349.10	185.04	73.10	114.92	16.40
Propanoic acid	0.43	0.71	0.43	2.93	0.87	-	1.10	0.48	-	7.07	7.65	0.94	14.39	22.71	-
Butanoic acid	10.88	5.70	4.23	10.48	8.37	19.13	15.04	9.36	20.17	4.91	14.94	20.07	4.68	8.52	3.43
Isovaleric acid	8.56	3.06	0.76	1.39	1.43	1.03	4.11	2.89	3.99	2.48	4.35	2.74	-	-	3.38
Pentanoic acid	0.68	0.78	0.59	1.08	0.87	0.68	1.07	0.91	1.17	1.21	2.90	1.11	0.95	2.82	1.00
Hexanoic acid	2.98	3.25	2.62	5.04	4.12	2.74	5.30	6.03	13.22	6.21	16.35	6.68	16.21	5.94	2.60
4-hexenoic acid	-	-	-	-	-	-	-	-	-	54.84	-	-	-	-	-
Heptanoic acid	0.89	1.18	0.74	1.32	1.08	0.66	1.41	0.98	1.45	1.60	2.22	1.32	1.30	1.86	1.06
Octanoic acid	2.55	3.14	2.65	6.45	4.63	2.63	3.84	3.89	6.19	7.34	5.93	4.90	4.57	6.79	2.76
Nonanoic acid	1.99	2.01	1.75	2.64	1.91	1.94	2.45	1.22	2.27	1.81	2.28	3.03	3.58	3.62	2.01
n-decanoic acid	1.82	2.98	2.56	6.68	5.18	2.35	5.58	2.52	4.38	7.37	5.86	5.09	4.84	6.10	1.79
Dodecanoic acid	1.70	4.24	1.99	8.87	1.99	2.23	4.81	0.89	2.36	4.10	1.66	1.87	1.50	2.67	0.84
Acids	**43.75**	**52.98**	**45.61**	**142.64**	**109.69**	**45.12**	**131.80**	**43.55**	**120.82**	**302.91**	**413.24**	**232.80**	**125.11**	**175.96**	**35.27**
Acetic acid, methyl ester	-	-	-	-	-	-	-	-	-	2.74	9.55	2.73	-	1.72	-
Ethyl Acetate	1.30	1.02	2.93	2.78	2.42	12.85	23.96	1.60	22.98	14.30	6.52	3.31	2.90	13.65	1.07
Butanoic acid, ethyl ester	-	-	0.33	0.91	0.72	4.71	1.94	0.78	5.68	0.90	0.97	0.46	1.00	8.84	-
Hexanoic acid, ethyl ester	2.58	1.80	1.73	1.79	1.76	6.97	4.17	2.53	17.18	2.72	1.71	-	-	7.58	-
4- Hexenoic acid, ethyl ester	-	-	-	-	-	-	-	-	-	37.68	-	-	-	-	-
Octanoic acid, ethyl ester	-	-	1.14	1.60	1.07	14.60	7.61	1.12	13.55	6.24	4.20	-	2.89	7.18	1.00
Dodecanoic acid, methyl ester	-	-	-	-	-	-	-	-	-	1.45	2.28	0.61	-	-	-
Dodecanoic acid, ethyl ester	1.75	-	1.35	1.84	1.03	13.02	7.95	0.62	6.98	4.65	2.56	-	2.63	9.86	-
Benzoic acid, ethyl ester	-	-	-	-	-	-	-	-	2.82	11.28	-	-	-	-	-
Esters	**5.62**	**2.82**	**7.48**	**8.93**	**7.00**	**52.15**	**45.64**	**6.65**	**69.20**	**81.96**	**27.79**	**7.11**	**9.42**	**48.82**	**2.07**
Spices															
Ethylene sulfide	-	-	-	89.60	46.31	-	-	-	-	6.04	60.08	20.89	-	20.45	48.28
Allyl methyl sulfide	1.33	-	-	11.88	8.60	-	6.72	-	0.71	4.36	12.68	28.77	-	4.66	46.80
Dimethyl disulfide	-	-	-	-	-	-	-	-	-	-	-	-	-	36.33	-
Diallyl sulfide	-	-	-	-	-	-	-	-	-	-	25.88	-	-	4.49	-
Diallyl disulfide	-	-	-	39.33	33.85	0.68	-	-	0.92	4.86	60.72	22.00	7.58	9.56	48.09
Garlic compounds	**1.33**	**-**	**-**	**140.81**	**88.76**	**0.68**	**6.72**	**-**	**1.63**	**15.26**	**159.36**	**71.66**	**7.58**	**75.49**	**143.17**
α-Pinene	31.94	21.25	2.42	12.63	9.91	8.08	27.47	1.49	4.5	-	-	1.54	-	-	16.51
β-Pinene	31.86	15.47	1.33	13.91	13.21	9.57	25.03	1.17	4.99	-	-	1.21	-	-	23.82
β-Phellandrene	100.36	54.25	3.82	25.24	17.98	36.93	45.89	3.05	14.53	-	-	2.16	-	-	13.42
α-Phellandrene	14.71	6.34	-	25.63	21.42	4.87	26.41	1.14	5.48	0.71	-	-	2.89	1.31	36.01
3-carene	26.00	9.64	-	62.75	59.17	20.26	58.11	-	14.94	-	-	-	-	-	34.20
β-Myrcene	59.91	32.93	5.86	46.22	36.95	26.22	54.16	2.63	4.40	-	-	-	-	-	37.50
β-pinene	3.65	1.65	-	5.32	4.63	2.14	4.72	-	-	-	-	-	-	-	-
4-carene	20.51	8.43	4.48	16.73	14.31	11.44	18.04	5.67	11.85	-	-	9.36	-	-	21.86

D-limonene	196.37	110.64	7.22	141.89	123.93	87.79	161.15	9.64	57.08	2.06	2.85	8.50	3.43	3.51	222.73
β-ocimene	8.53	4.29	0.34	2.66	2.19	1.03	4.73	-	0.73	-	-	-	-	-	2.10
3-carene	6.47	2.68	5.35	2.14	1.70	6.49	5.22	2.96	2.81	0.27	-	7.52	-	-	2.46
p-cymene	13.35	6.63	5.46	14.28	12.53	14.26	13.75	3.76	8.97	-	-	-	-	-	20.31
cis- β-Terpineol	2.26	1.30	0.49	0.39	0.38	2.43	1.82	0.18	1.55	-	-	1.77	-	-	-
α-Cubebene	2.25	0.95	-	1.15	1.13	1.84	1.89	0.67	1.82	-	-	-	-	-	1.90
Copaene	21.52	7.96	1.07	8.71	8.76	10.94	7.66	2.37	11.04	-	-	1.94	1.27	-	11.78
Linalool	7.80	3.13	23.89	4.39	3.07	4.41	2.88	0.96	3.80	-	-	2.59	-	-	4.55
cis- β-Terpineol	2.20	1.91	0.73	0.88	0.66	3.05	1.96	0.53	2.07	-	-	3.99	-	-	-
3,5-octadien-2-one	-	-	-	-	-	-	-	-	-	-	-	-	1.45	7.27	-
trans- α-bergamotene	7.66	5.74	-	-	-	0.64	4.75	-	-	-	-	-	-	-	-
Caryophyllene	149.65	52.03	6.28	70.26	68.19	37.68	65.75	11.90	54.73	-	-	7.18	2.52	-	84.85
Terpenes	**707.00**	**347.22**	**68.74**	**455.18**	**400.12**	**290.07**	**531.39**	**48.12**	**205.29**	**3.04**	**2.85**	**47.76**	**11.56**	**12.09**	**534.00**
Safrole	-	-	-	-	-	45.82	45.90	-	1.79	-	-	-	-	-	-
Methyl eugenol	-	-	-	-	-	8.94	-	-	0.69	-	-	-	-	-	-
Eugenol	-	-	18.11	0.89	0.76	2.80	1.26	-	0.81	1.46	-	1.77	-	-	-
Phenyl propenes	**-**	**-**	**18.11**	**0.89**	**0.76**	**57.56**	**47.16**	**-**	**3.29**	**1.46**	**-**	**1.77**	**-**	**-**	**-**
Total	**708.33**	**347.22**	**86.85**	**596.88**	**489.64**	**348.31**	**585.27**	**48.12**	**210.21**	**19.76**	**162.21**	**121.19**	**19.14**	**87.58**	**677.17**
Smoking VOCs															
2-furanmethanol	-	-	-	7.26	7.02	-	-	-	-	6.16	2.85	1.52	2.28	51.82	-
Furfural	-	-	-	-	-	-	-	-	-	-	-	-	-	4.71	-
2-methoxy-Phenol	-	-	-	7.06	5.90	-	0.48	0.57	-	13.42	8.04	4.75	1.57	87.49	-
2-methoxy-4-methyl-Phenol	-	-	-	2.84	2.42	-	-	-	-	4.16	2.85	1.99	-	28.91	-
2-methyl-Phenol	-	-	-	3.24	2.66	-	-	-	-	3.09	2.68	1.60	0.91	20.04	-
Phenol	-	-	-	11.63	7.35	-	-	-	-	8.30	5.51	2.89	2.10	52.45	-
4-ethyl-2-methoxy-Phenol	-	-	-	-	-	-	-	-	-	2.08	2.01	1.52	-	10.07	-
2,4-dimethyl-Phenol	-	-	-	1.26	1.27	-	-	-	-	1.93	-	-	-	7.60	-
4-methyl-Phenol	0.64	-	0.54	2.32	1.95	1.50	-	-	1.25	3.42	1.81	1.95	1.01	13.92	-
3-methyl-Phenol	-	-	-	3.30	2.67	1.09	-	-	-	3.68	2.57	1.85	-	18.97	-
2,6-dimethoxy-Phenol	-	-	-	-	-	-	-	-	-	-	-	-	-	9.90	-
Total	**0.64**	**-**	**0.54**	**31.64**	**24.23**	**2.59**	**0.48**	**0.57**	**1.25**	**40.07**	**25.48**	**16.55**	**5.59**	**249.35**	**-**

*not detected under the adopted conditions

[§]: samples subjected to a smoking phase

[†]: samples added with smoked paprika

Table 3.7 also reports VOCs derived from the microbial biochemical activities, that occurred during sausage fermentation and ripening. The compounds are grouped into chemical classes, whose total amounts are shown in Figure 3.7.

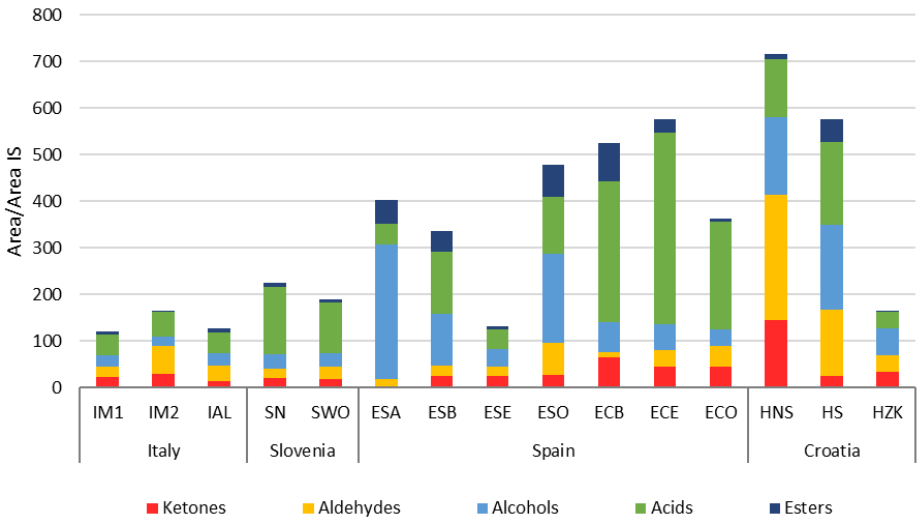


Figure 3.7: Presence of the different classes of volatile organic compounds (VOCs) derived from the microbial biochemical activities in the fifteen fermented sausage samples considered. The values are expressed as the ratio between the peak area of the compound considered and the area of the internal standard.

Higher quantities of VOCs were found in some Spanish products and two Croatian samples. Ketones were evenly distributed in the analysed samples, except for chorizo and HNS products, in which their total amount was higher. On the other hand, lower values were found in salchichón ESA. These molecules mostly derive from fatty acid oxidation and, in particular, from β-oxidation. Some microbial groups such as staphylococci and fungi can have a role in these phenomena. It is interesting to observe that products characterised by high concentrations of fat (for example HNS) showed the higher presence of ketones. HS fermented sausages, having the same formulation of HNS but subjected to smoking, presented lower ketone amounts: this can be attributed to the antioxidant role of some of the compounds produced by smoking treatment. It is also interesting to note that chorizo samples are characterised by higher ketones amounts among Spanish products. These fermented sausages did not contain nitrates or nitrites, which exert a well-known antioxidant activity, while these preservatives were present in salchichón formulations.

Among ketones, 2-butanone prevailed in some samples, *i.e.* ECB and HNS (Table 3.7). The presence of this molecule in fermented meat products is common, but its contribution to aroma profile can be negative depending on its amounts and the balance with other VOCs (Tabanelli et al., 2013). Diacetyl (2,3-butanedione) and acetoin (3-hydroxy-2-butanone) were particularly present in some Spanish products (ECB and ECO), in Slovenian samples and in two Croatian fermented

sausages (HNS and HZK). Diacetyl and acetoin are mainly produced through the catabolism of pyruvic acid by LAB (Flores, 2018).

Aldehydes were particularly relevant in the Italian product IM2 and in Spanish salchichón ESO, but the maximum amounts were found in Croatian HNS and HS. Most of the detected aldehydes can derive from fatty acids oxidation. Within certain limits, these compounds can contribute to the typical fermented sausages aroma profile. Nevertheless, given their strong aroma perception characterised by herbaceous notes, excessive quantities can lead to organoleptic defects, such as rancidity (Flores, 2018). Hexanal, together with nonanal, was certainly the most characteristic molecule of this VOC group. On the other hand, the methyl-branched aldehydes, such as isovaleric aldehyde (3-methyl-butanal), have often been associated with fermented sausage aroma and can derive from the bacterial metabolism of branched amino acids, in particular leucine (Carballo, 2012). Benzaldehyde and benzeneacetaldehyde were detected in most of samples. In particular, HS contained higher amounts of benzeneacetaldehyde, while benzaldehyde was predominant in HNS and ECO. These VOCs are the result of aromatic amino acid (phenylalanine and tyrosine) metabolism and can contribute to the product flavour, imparting floral and almond notes (Smid and Kleerebezem, 2014).

Alcohols were present in all the samples, but their amounts were higher in HNS and HS and some Spanish products, particularly in ESA and ESO samples (Table 3.7). Among alcohols, ethanol was the most abundant in analysed samples, with high amounts in some Spanish salchichón (ESB, ESA and ESO) and in Croatian products, while a lower presence was highlighted in Italian and Slovenian samples. The presence of this compound can be influenced by the wine addition in meat batter formulation, or it can be the result of several microbial pathways, *i.e.* pyruvate or amino acid metabolisms, being therefore strongly influenced by the natural microbiota composition of each product (Carballo, 2012). Phenethyl alcohol, which is the results of benzenacetaldehyde reduction and that can give a rose odour, was present in significant amounts in HS and ESA, samples characterised by high amounts of its precursor.

Acids prevailed in chorizo samples, but they were the most represented molecular class in Slovenian sausages (Figure 3.7). Acetic acid was found in low amounts in Italian products and in ESA, ESE and HZK samples, which showed high pH values. In contrast, significantly higher amounts of acetic acid were detected in chorizo samples, particularly in ECE. These samples had the lowest pH, ranging from 4.52 to 5.04. Acetic acid can be produced, similarly to ethanol, with many bacterial metabolic pathways and different microbial groups can be responsible for its accumulation, including LAB, staphylococci and fungi. It is interesting to underline the presence of isovaleric acid (3-methyl, butanoic acid) (Table 3.7), whose occurrence in fermented sausages is

well-documented and which can exert a very strong organoleptic impact even in low quantities (Montanari et al., 2021a). Higher amounts of this VOC were detected in IM1 sample, while it was not present in HS and HNS products.

Esters were present in lower amounts in comparison to other VOC classes and were mainly represented by ethyl acetate, ethyl hexanoate and ethyl octanoate (Table 3.7). Their levels showed dependence on fermented sausage type. They were more abundant in some Spanish samples and in HS, being related to the presence of their precursors and to the esterase activities typical of the microbial communities forming their microbiome.

3.1.4 Conclusions

The analysis of the microbial communities associated with traditional spontaneous fermented meat products highlighted the high variability in the qualitative and quantitative composition of the microbiota involved in these natural fermentations. LAB and CNC were the most representative microorganisms in all the samples. However, their relative ratio drastically changed. In addition, within each group, the relative presence of species and genus was extremely different. In this context, LAB were characterised by high biodiversity, and *Latilactobacillus* was the only genus found in all the products. The great LAB biodiversity can derive from both meat and the production environment, that can affect the growth and survival of the different microbial groups.

These differences are reflected in the first instance in fermented sausages safety characteristics, *i.e.* biogenic amines concentrations. Moreover, also the volatilome, and consequently the peculiar sensory features of traditional products, were dependent on the complexity of the microbiota. In many analysed products, the increase in microbial biodiversity led to a higher complexity in VOCs composition, both qualitatively and quantitatively. Therefore, a deep knowledge of the peculiar characteristics of traditional fermented sausages can valorise these niche productions, guaranteeing their recognisability.

In conclusion, the biodiversity highlighted the possibility to exploit these products to find new strain candidates to be used as autochthonous starter cultures or bioprotective agents in meat products. Indeed, these traditional spontaneous fermented sausages represented an important source of isolation of indigenous LAB strains, belonging to different species, that in the further phase of this PhD thesis were studied for their features, considering both safety and technological aspects, in order to select the most promising for food applications. Being highly adapted to specific ecological niches, they can be successfully used in traditional meat products to control undesirable microbiota,

also avoiding the accumulation of biogenic amines, and/or to endow the final product with peculiar aromatic characteristics.

Data published as:

Barbieri, F., Tabanelli, G.*, Montanari, C., Dall'Osso, N., Šimat, V., Smole Možina, S., Baños, A., Özogul, F., Bassi, D., Fontana, C., Gardini, F. (2021). Mediterranean spontaneously fermented sausages: spotlight on microbiological and quality features to exploit their bacterial biodiversity. *Foods*, 10, 2691. doi:10.3390/foods10112691.

3.2 Taxonomical identification and safety characterisation of *Lactobacillaceae* isolated from Mediterranean natural fermented sausages

3.2.1 Introduction

The curing of meats can be considered one of the most ancient methods for preserving perishable raw materials (Franciosa et al., 2018). Among these products, dry sausage preparation combines the use of curing salts with a fermentation step that involves several microorganisms including LAB, staphylococci, micrococci and fungi (Kumar et al., 2015). This complex dynamic of microbial population leads to establish criteria for the selection of specific starter cultures to be used for driving meat fermentation, depending on the type of raw material and the process parameters employed (Comi et al., 2020). Nowadays, the use of starter cultures is common in industrial products, and they are mainly constituted of LAB and coagulase-negative cocci (CNC). Among LAB, the strains mainly selected belong to the species *Lat. sakei*, *Lat. curvatus*, *Ltp. plantarum*, *Pediococcus pentosaceus* and *P. acidilactici* (Cocconcelli and Fontana, 2014). However, as observed by Franciosa et al. (2018), the use of starter cultures could result in the loss of microbial biodiversity and, therefore, of the peculiar characteristics of traditional products (impoverishment of sensory features), if compared to artisanal sausages obtained with spontaneous fermentation. On the other hand, selected cultures are able to guarantee a constant quality, safety and prolong shelf-life of these products (Carballo, 2021).

The research for new and tailor-made cultures able to confer specific and traditional attributes to fermented sausages represents an important approach to overcome the negative aspects and preserving the authenticity and recognisability of artisanal products. In this perspective, the presence of numerous local products, still obtained through spontaneous fermentation, is an important source of unexplored microbial biodiversity of a given territory, which could be exploited for isolating new starter candidates (Van Reckem et al., 2019).

The main roles of LAB strains in meat fermentation are certainly their abilities to rapidly decrease the pH and to colonise the environment throughout the entire production process (Flores, 2018). A low pH limits the growth of undesirable spoilage and pathogenic microorganisms and favours texture and water loss by approaching the isoelectric point of meat proteins (Ameer et al., 2021). However, further technological and functional characteristics are requested for their use as meat starter cultures. These features can represent an important issue for starter cultures selection, also due to the new market trends regarding the NaCl and synthetic additives reduction, considering nutritional needs and consumer demands.

Generally, LAB starters are selected in order to improve safety and reduce hygienic and toxicological risks in foods, but they have to be firstly safe, so that their use as starter cultures for guide fermentation in food industry requires a safety assessment (Franciosa et al., 2018).

Biogenic amines (BA) are toxic products, especially tyramine and histamine, deriving from amino acid decarboxylation, that accumulate in sausages during fermentation and ripening due to the metabolisms of several microorganisms, including LAB (Barbieri et al., 2019). For this reason, the absence of specific decarboxylases is a prerequisite for LAB used as starter cultures. The amino biogenic potential of strains is linked to the presence of genetic clusters containing the necessary genes for BA production, that can be tested both genetically and phenotypically (Dos Santos Cruxen et al., 2019).

Another relevant safety aspect is the presence of antibiotic-resistance genes in mobile genetic elements, such as plasmids and transposons. In fact, these elements can be transferred to other species, including pathogenic microorganisms, during food manufacture or during the passage through the gastrointestinal tract. This poses an additional risk due to the nature of consumption of RTE fermented products and their potential to become strong antibiotic-resistance reservoirs (Daza et al., 2022). In particular, the presence of tetracycline and erythromycin resistant lactobacilli was well documented in fermented dry sausages produced in northern Italy (Fontana et al., 2021), according to EFSA indications (EFSA Panel, 2012).

In this part of PhD thesis, the identification, characterisation and safety assessment of autochthonous LAB, isolated from the fifteen Mediterranean spontaneously fermented sausages analysed in Chapter 3.1, were performed. This investigation had the purpose to widen the previously studied microbiota composition, in order to understand the ecology of these natural fermented meat products and to know which LAB species are the most abundant and persistent. With this aim, more than 900 isolates have been genotyped using fingerprint analysis for the differentiation of the strains, which were further taxonomically identified and characterised firstly for their safety features. In particular, the identified strains were studied regarding their antimicrobial resistances and amino biogenic potential.

This knowledge will be the starting point to further determine strains suitability to be used in foods as potential autochthonous starter cultures, studying their technological properties, and secondary, their protective features against spoilage microorganisms and food-borne pathogens, assessing their antimicrobial potential.

3.2.2 Materials and methods

3.2.2.1 LAB isolation and purification obtained from spontaneously fermented sausages samples

As reported in Chapter 3.1, fifteen natural-fermented sausages, produced in different Mediterranean countries (Table 3.1) without any starter's addition, were previously characterised at the end of ripening for their physico-chemical and microbial aspects.

Isolates were obtained from purification of colonies representative of the different morphologies into MRS plates obtained from the microbial characterisation of these samples. Starting by plates containing from 20 to 50 LAB colonies, they were randomly selected, picked with a sterile loop and streaked onto new MRS plates in duplicate. This procedure was repeated until obtained a pure isolate. A minimum of 22 to a maximum of 70 of presumptive LAB isolates for each sample was achieved. They were then observed for morphological characteristics and tested by means of catalase tests and Gram staining. For their further taxonomical identification and to perform other analyses, the isolates were stored at -20°C in MRS broth containing 20% glycerol (Carlo Erba, Milan, Italy).

3.2.2.2 DNA extraction and rep-PCR analysis to isolates identification

Presumptive LAB isolates were subjected to genotypic characterisation through repetitive element palindromic PCR analysis (rep-PCR) and taxonomic identification by 16S rRNA gene sequencing.

All isolates were inoculated in 10 ml of MRS broth and incubated at 30°C overnight. After their growth, they were streaked in MRS agar and again incubated at 30°C for 48 h. Single colonies were selected from the agar plates to perform DNA extraction using the fast microLYSIS®-Plus DNA extraction kit (Microzone, Labogen, Stourbridge, UK), according to the protocol described by the manufacturer.

Firstly, LAB differentiation was performed by rep-PCR fingerprinting by using primer (GTG)5 5'-GTGGTGGTGGTGGTG-3', in order to achieve fingerprint profiles of isolates (Dentice Maidana et al., 2020). Fingerprint profiles were evaluated for each group of isolates from different fermented sausages. PCR-products were separated by electrophoresis on a 1.5% agarose gel. For cluster analysis of rep-PCR electrophoretic profiles, digitized images were converted, and analysed with the software package Gelcompar II 4.0. To cluster the isolates, the levels of similarity between different electrophoretic profiles were computed using the Pearson correlation coefficient. Subsequently, 16S rRNA gene sequencing was performed on selected isolates present in each cluster, characterised by a different rep-PCR profile, through specific primers and PCR reaction

(Dentice Maidana et al., 2020). After amplification and before sequencing, PCR products were purified using ExoSAP – IT ™ (Applied Biosystems™; ThermoFisher Scientific) according to the protocol described by the manufacture. The DNA was sequenced by a commercial facility (Eurofins Genomics, Italy) and the obtained sequences were analysed using the Ribosomal Database Project tools (http://rdp.cme.msu.edu/, accessed on 1 February 2021) and assigned to the species with the highest percentage of identity.

Moreover, to establish the taxonomic identification of the isolates that were not correctly assigned to species level (identity ≤ 98.7%), species-specific PCR reactions were performed using specific primers for the detection of *Lat. sakei* and *Lat. curvatus*, since they represent the main species colonising fermented meat (Chun et al., 2018). PCR products were separated by electrophoresis in a 1% agarose gel and visualised by Sybr-Safe staining. The relative frequency of intra-species biotypes for each MC salami sample has been calculated using Microsoft Excel 2016, Version 2207.

3.2.2.3 Antibiotic-resistance profile determination and PCR-based screening of resistance genes

Antibiotic-resistance determination of selected strains, one representative of each previously determined cluster, was assessed following EFSA indications (EFSA Panel, 2012). A minimal inhibitory concentration (MIC) test to evaluate the resistance to ampicillin (Amp), chloramphenicol (Chl), clindamycin (Cli), erythromycin (Ery), gentamicin (Gen), tetracycline (Tet), kanamycin (Kan) and streptomycin (Str) was performed by using micro dilution technique in the recommended LSM medium (Iso-Sensitest™ broth 90% and MRS broth 10%; ThermoFisher Scientific). The results were collected after 48 h of incubation at 30°C. Relative abundance of resistant biotypes for each species and antibiotics tested were calculated using Microsoft Excel 2016, Version 2207.

Moreover, the presence of tetracycline and erythromycin resistance genes was screened by standard PCR with specific primers reported in Table 3.8. Genes coding for ribosomal protection proteins conferring tetracycline resistance were targeted with specific primers for *tetW*, *tetM* and *tetS*. Tetracycline efflux pump gene, *tetL*, was also detected using its gene-specific primers. The presence of erythromycin resistance genes was tested using specific primers for *ermA*, *ermB* and *ermC*. PCR products were separated by electrophoresis on a 1.5% agarose gel and visualised by Sybr-Safe staining.

Table 3.8: Selected primers to research tetracycline and erythromycin antibiotic-resistance genes.

Primer's name	Oligonucleotides sequence (5'-3')	Expected band (bp)	Positive control strains
ermA1 **ermA2**	TCTAAAAAGCATGTAAAAGAA CTTCGATAGTTTATTAATATTAGT	645	*Ent. faecium* PE1
ermB1 **ermB2**	GAAAAGGTACTCAACCAAATA AGTAACGGTACTTAAATTGTTTAC	639/694	*Ent. faecium* PE1
ermC1 **ermC2**	ATCTTTGAAATCGGCTCAGG CAAACCCGTATTCCACGATT	275/294	*Limosilactobacillus reuteri* 70
tetL1 **tetL2**	GTMGTTGCGCGCTATATTCC GTGAAMGRWAGCCCACCTAA	696	*Ent. faecium* LMG 20927
tetM1 **tetM2**	GAACTCGAACAAGAGGAAAGC ATGGAAGCCCAGAAAGGAT	740	*Ltp. plantarum* 146
tetS1 **tetS2**	GGAGTACAGTCACAAACTCG GGATATAAGGAGCAACTTTG	335	*Lim. reuteri* 541
tetW1 **tetW2**	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	168	*Lim. reuteri* 534

3.2.2.4 Assessment of biogenic amines strains production

The amino biogenic potential of selected biotypes was tested through the screening in Bover-Cid-Holzapfel medium (BC). All strains were pre-cultivated in MRS broth and then inoculated in BC broth, supplemented with the BA precursors (histidine, tyrosine, ornithine or lysine) and incubated at 30°C (Bover-Cid and Holzapfel, 1999). After that, the supernatants of presumptive positive strains were collected and stored at -20°C. Samples were derivatised and injected into HPLC for their BA production confirmation (Paragraph 3.1.2.5).

3.2.3 Results and discussion

3.2.3.1 Strain genotyping and identification of biotypes

The spontaneously fermented sausages used as a source of isolation of new LAB strains were produced according to traditional local recipes and varied with regards to ingredients, the presence of additives, the casing, the fat, and the ripening conditions as previously described (Chapter 3.1).

Afterwards, a total of 914 microorganisms, grown on MRS agar medium and presumptive classified as LAB based on Gram staining and catalase test results, were isolated from these natural fermented sausages (Table 3.1). In particular, 173 isolates from Italian sausages, 140 from Slovenian sausages, 444 from Spanish sausages and 157 from Croatian sausages were obtained. To achieve taxonomical identification at the strain level, (GTG)5-rep-PCR fingerprinting technique was applied on DNA extracted from the all the isolated samples. Representative profiles for each sausage were selected and subjected to partial 16S rRNA gene sequencing and species-specific PCR for *Lat. sakei* and *Lat. curvatus*. In Table 3.9 the total number of isolates (914) and biotypes detected (151) for each type of Mediterranean spontaneously fermented sausage are reported.

Table 3.9: Number of isolates and biotypes detected for each type of Mediterranean spontaneously fermented sausage.

Production Countries	Products (source of isolation)	Products name	Number of isolates	Biotypes
Italy	Salame Fabriano_product 1 (Marche region)	IM1	58	3
	Salame Fabriano_product 2 (Marche region)	IM2	48	2
	Salame Alfianello (Lombardia region)	IAL	67	4
Slovenia	Traditional smoked salami with nitrates	SN	70	5
	Traditional smoked salami without nitrates	SWO	70	6
Spain	Salchichón Alhendín	ESA	67	11
	Salchichón Bérchules	ESB	69	11
	Salchichón Écija	ESE	69	8
	Salchichón Olvera	ESO	70	19
	Chorizo Bérchules	ECB	48	16
	Chorizo Écija	ECE	69	22
	Chorizo Olvera	ECO	52	7
Croatia	Traditional unsmoked salami	HNS	55	11
	Traditional smoked salami	HS	49	5
	Salami Zminjska Klobasica	HZK	53	21
Total	**15**		**914**	**151**

The fingerprint analysis showed a higher variability in the electrophoretic profiles of samples from Spanish and Croatian sausages. In particular, starting from 69 and 70 isolates of the Spanish salchichón ESB and ESO, 11 and 19 different biotypes were respectively differentiated, while 16 and 22 biotypes were detected in Spanish Chorizo ECB and ECE, characterised by 48 and 69 isolates respectively. Regarding Croatian samples, the richest in LAB biodiversity was the sample HZK, with 21 biotypes out of 53 isolates. Conversely, Italian and Slovenian fermented sausages were characterised by a lower biodiversity, with only 2 biotypes identified among 48 isolates from Italian product IM2, 5 biotypes among 70 isolates from the Slovenian traditional smoked salami SN and 6 biotypes among 70 isolates from the Slovenian traditional smoked salami SWO.

Secondarily, the 151 strains that showed unique rep-PCR profiles, were identified by 16S rRNA gene sequencing. Isolates that failed to be assigned to any species (level of identity ≤ 98.7%) were identified using species-specific PCR for the identification of *Lat. sakei* and *Lat. curvatus*. The combined molecular approaches allowed to detect three dominant species: *Lat. sakei* (90), *Lat. curvatus* (40) and *Comp. alimentarius* (21). As frequently stated by previous works (Casaburi et al., 2016), natural meat fermentation is dominated by coagulase-negative cocci (CNC) and LAB, whose most commonly species are represented by *Lat. sakei*, *Lat. curvatus* and *Ltp. plantarum*. Considering the result of the combined methodology to assess the taxonomic identity of the 151 LAB biotypes, *Lat. sakei* resulted to be the dominant species in IM1 (100%), IM2 (100%), IAL (75%), SN (100%), SWO (100%), ESB (91%), ESA (82%), ESE (62.5%), HZK (81%), HS (100%),

HNS (64%) products. *Lat. curvatus* prevailed in ECE (63.7%) sample, while ESO fermented sausage showed an equal presence of *Lat. sakei* and *Lat. curvatus* (both 47%). Finally, *Comp. alimentarius* dominated in ECB (87.5%) and in ECO (57%) chorizo products (Figure 3.8).

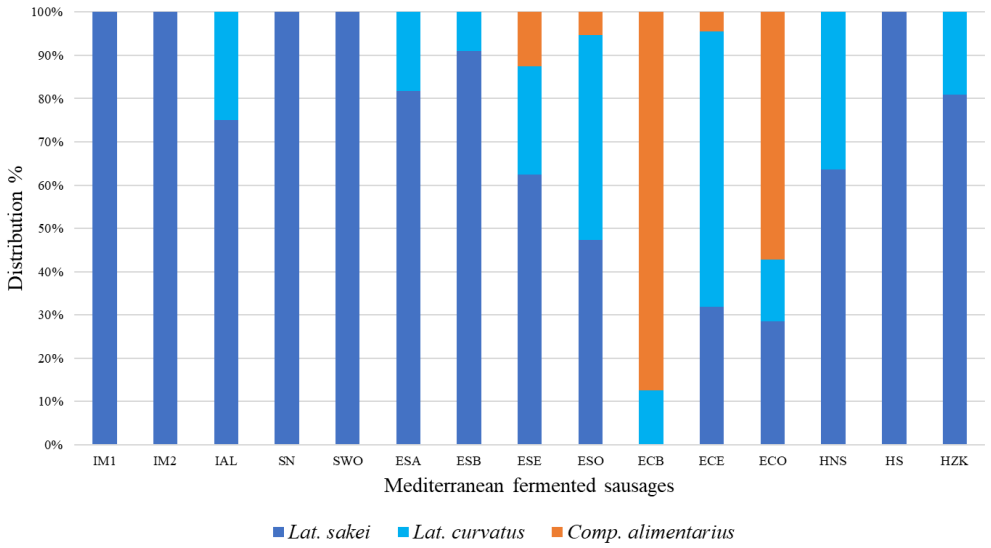


Figure 3.8: Distribution of the species detected in different spontaneously fermented sausages used as source of LAB isolation.

These data confirmed the result obtained through metagenomics analysis on the same samples, where *Lat. sakei* was the dominant species among LAB, especially in IM2, IAL, SN, SWO and HS products (Figure 3.4). This dominance was highlighted also by the relative frequency of *Lat. sakei* biotypes, that were found in higher percentage particularly in Italian and Slovenian fermented sausages, followed by Croatian ones. On the other hand, a higher species biodiversity was commonly found into Spanish samples, in which their distribution among biotypes was more balanced for the three described LAB (Figure 3.9).

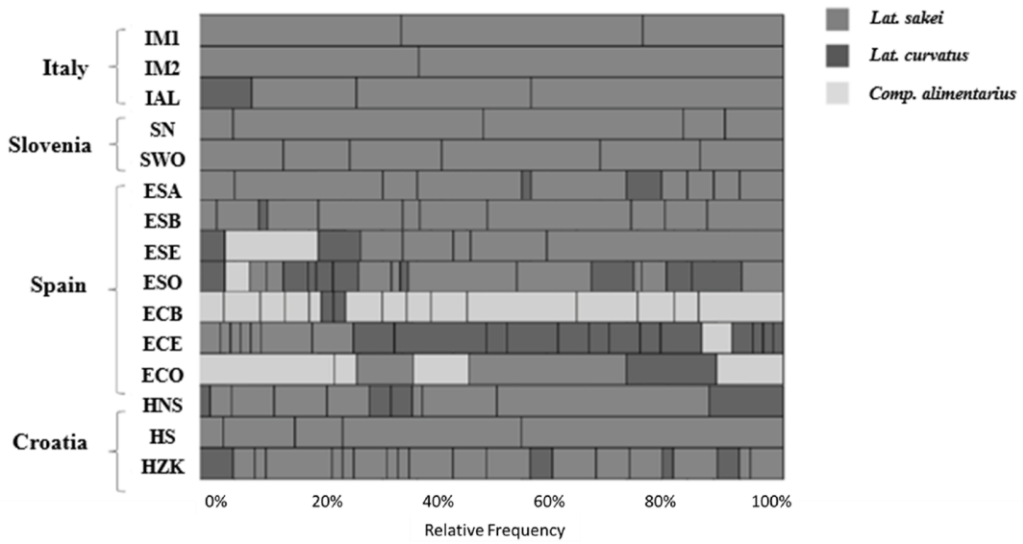


Figure 3.9: Relative frequency of LAB biotypes identified as *Lat. sakei*, *Lat. curvatus* and *Comp. alimentarius*, present in each Mediterranean fermented sausage.

During the ripening process of natural fermented sausages, LAB species diversity is limited and *Lat. sakei* is one of the main adapted species to the restrictive conditions generally present in the dry meat environment, due to the species excellent adaptation, competitiveness and assertiveness in the meat matrix (McLeod et al., 2017; Montanari et al., 2018b). Regarding these analyses, samples were processed at the end of the maturation period when low availability of sugars was still supposed to be present in the sausages and free amino acids were probably used by *Lat. sakei* to grow and survive in these conditions. This species is highly adapted to this ecological niche, due to its metabolic pathways, including the arginine deiminase pathway and the utilisation of nucleosides (Janßen et al., 2018; Montanari et al., 2018b; Widenmann et al., 2022).

However, Spanish fermented sausages surprisingly showed a high percentage of biotypes belonging to *Comp. alimentarius* species, particularly in ECB sample (87.5%). This species was previously highlighted through amplicon sequencing and metagenomic analysis in these Mediterranean fermented sausage samples (Paragraph 3.1.3.4). In fact, with these preliminary analyses, high quantities of members of *Comp. alimentarius*, *Comp. heilongjiangensis* and *Comp. versmoldensis* were detected in many Spanish products, in particular ESE and ECB (55.3% and 45% of the total ASVs, respectively). This species was reported in the literature as a regional peculiarity (Fontán et al., 2007a,b), but its presence was described also as minoritarian in some traditional fermented salami of Southern Italy, such as Naples-type salami (Coppola et al., 2000).

In addition, the Spanish samples, if compared to the other Mediterranean fermented meats, had a higher strain biodiversity in terms of different identified biotypes. Technological parameters, together with ingredients, the fermentation process and ripening conditions could strongly influence the survival and adaptation of different microbial populations to a peculiar environment.

3.2.3.2 Safety assessment of isolated strains

Potential candidate strains to be used in food industry as starter or bio-protective cultures must fulfil safety criteria, such as the absence of antibiotic-resistance genes and the incapacity to produce biogenic amines (Coton et al., 2018). In this context, the two main occurring species, represented by *Lat. sakei* and *Lat. curvatus* (90 and 40 isolates, respectively), were tested for their safety features in order to prove their safe use as food cultures (Table 3.10). In particular, their antimicrobial-resistance profile was tested by microdilution technique and the amino biogenic potential through HPLC analysis. *Comp. alimentarius* strains were not screened for these analyses, since this species is not considered among the possible adequate starter cultures in meat productions.

Table 3.10: List of strains tested for their safety aspects, grouped for each product.

Production Countries	Products (source of isolation)	Samples name	Species	List of biotypes
Italy	Salame Fabriano_product 1 (Marche region)	IM1	*Lat. sakei*	1M8, 1M24, 1M51
	Salame Fabriano_product 2 (Marche region)	IM2	*Lat. sakei*	2M7, 2M9
	Salame Alfianello (Lombardia region)	IAL	*Lat. sakei*	IAL8, IAL18, IAL38
			Lat. curvatus	IAL6
Slovenia	Traditional smoked salami with nitrates	SN	*Lat. sakei*	SN4, SN34, SN58, SN63, SN70
	Traditional smoked salami without nitrates	SWO	*Lat. sakei*	SWO10, SWO18, SWO29, SWO48, SWO60, SWO61
Spain	Salchichón Alhendín	ESA	*Lat. sakei*	SA4, SA21, SA25, SA37, SA49, SA56, SA59, SA62, SA63
			Lat. curvatus	SA38, SA53
	Salchichón Bérchules	ESB	*Lat. sakei*	SB2, SB7, SB14, SB24, SB26, SB34, SB53, SB57, SB60, SB67
			Lat. curvatus	SB8
	Salchichón Écija	ESE	*Lat. sakei*	SE24, SE30, SE32, SE41, SE67
			Lat. curvatus	SE3, SE19
	Salchichón Olvera	ESO	*Lat. sakei*	SO8, SO10, SO23, SO24, SO38, SO47, SO53, SO56, SO65
			Lat. curvatus	SO6, SO13, SO14, SO16, SO19, SO25, SO52, SO59, SO61
	Chorizo Bérchules	ECB	*Lat. curvatus*	CB11, CB12
	Chorizo Écija	ECE	*Lat. sakei*	CE2, CE3, CE4, CE5, CE6, CE10, CE15
			Lat. curvatus	CE16, CE25, CE27, CE32, CE35, CE37, CE40, CE42, CE46, CE51, CE52, CE53, CE54, CE57
	Chorizo Olvera	ECO	*Lat. sakei*	CO19, CO38
			Lat. curvatus	CO46
Croatia	Traditional unsmoked salami	HNS	*Lat. sakei*	KN3, KN7, KN12, KN16, KN21, KN28, KN48
			Lat. curvatus	KN1, KN18, KN20, KN55
	Traditional smoked salami	HS	*Lat. sakei*	KS1, KS2, KS8, KS13, KS42
	Salami Zminjska Klobasica	HZK	*Lat. sakei*	ZK5, ZK6, ZK10, ZK11, ZK12, ZK15, ZK18, ZK19, ZK23, ZK26, ZK30, ZK36, ZK39, ZK42, ZK47, ZK50, ZK53
			Lat. curvatus	ZK3, ZK32, ZK43, ZK49
Total strains				**130**

- Antibiotic-resistance assessment

Cut-off values established by EFSA (EFSA Panel, 2012) were used as references to evaluate the presence of resistant biotypes isolated from the 15 artisanal fermented sausages. A unimodal distribution of MIC values, divided per species, is reported in Table 3.11 for all the analysed samples.

The highest MIC values were detected against kanamycin and streptomycin antibiotics in strains isolated from ESA, HS and HZK samples and ESE, ESO, ECE, HNS and HZK products, respectively. Concentration of 64 µg/ml was reported for gentamicin (ESA and ESE) and tetracycline (ESB, ESO, ECEO, ECO, HNS and HZK), followed by 8 µg/ml MIC value for clindamycin (HS), chloramphenicol (ECE and HZK) and ampicillin (ESO and HNS). Finally, strains resistant to erythromycin were characterised by the highest MIC value of 2 µg/ml (ESO and HZK).

Table 3.11: Unimodal distribution of MIC for *Lat. sakei* and *Lat. curvatus* isolated biotypes. The number of resistant strains for each antibiotic are highlighted according to the cut offs reported by EFSA (EFSA Panel, 2012) (bold and italics).

Antibiotics[a]	Species	MIC values (µg/ml)														
		<0.016	**0.032**	**0.063**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
Gen	*Lat. sakei*						1	1	4	11	33	27	***10***	***3***		
	Lat. curvatus						1	3	2	9	10	10	***5***			
Kan	*Lat. sakei*								1	5	12	20	20	21	***5***	***6***
	Lat. curvatus								1	3	8	11	7	7	***3***	
Str	*Lat. sakei*								1	5	10	10	17	22	***22***	***3***
	Lat. curvatus							1		2	3	7	7	9	***4***	***7***
Tet	*Lat. sakei*				7	5	9	26	17	5	6	***1***		***14***		
	Lat. curvatus					2	4	3	9	9	6	***6***	***1***			
Ery	*Lat. sakei*	4	11	37	17	13	3	5								
	Lat. curvatus		3		7	15	7	6	***2***							
Clin	*Lat. sakei*		66	6	1	9	2	2	***2***	***1***	***1***					
	Lat. curvatus		20	3	11	1		2	***2***	***1***						
Chlor	*Lat. sakei*				7	2	10	33	29	7	***2***					
	Lat. curvatus						1	4	4	27	***3***	***1***				
Amp	*Lat. sakei*		7			2	9	20	12	40						
	Lat. curvatus					1	15	11	7	4	***2***					

[a]Gen = Gentamicin; Kan = Kanamycin; Str = Streptomycin; Tet = Tetracycline; Ery = Erythromycin; Clin = Clindamycin; Chlor = Chloramphenicol; Amp = Ampicillin.

The collected data underlined the fact that the occurrence of strains characterised by MIC value higher than EFSA breakpoints was found to be superior in the ones isolated from Spanish and Croatian fermented sausage samples, showing a geographical distribution of resistant biotypes. In fact, all Italian and Slovenian strains were sensitive to all the tested antibiotics (Table 3.12 and Table 3.13).

Table 3.12: *Lat. sakei* antibiotic-resistance.

Fermented sausages	*Lat. sakei* strains	Gen	Kan	Str	Tet	Ery	Clin	Chlor	Amp
1M	1M8, 1M24, 1M51	S	S	S	S	S	S	S	S
2M	2M7, 2M9	S	S	S	S	S	S	S	S
IAL	IAL8, IAL18, IAL38	S	S	S	S	S	S	S	S
SN	SN4, SN34, SN58, SN63, SN70	S	S	S	S	S	S	S	S
SWO	SWO10, SWO18, SWO29, SWO48, SWO60, SWO61	S	S	S	S	S	S	S	S
ESA	SA21, SA49	S	S	S	S	S	S	S	S
	SA4	**R**	S	S	S	S	S	S	S
	SA25	S	S	**R**	S	S	S	S	S
	SA37	S	S	**R**	S	S	S	S	S
	SA56	S	S	**R**	S	S	S	S	S
	SA59	S	S	**R**	S	S	S	S	S
	SA62	**R**	**R**	**R**	S	S	S	S	S
	SA63	**R**	**R**	**R**	S	S	S	S	S
ESB	SB2, SB7, SB14, SB24, SB53, SB60, SB67	S	S	S	S	S	S	S	S
	SB26	S	S	**R**	S	S	S	S	S
	SB34	S	S	**R**	S	S	S	S	S
	SB57	S	S	S	**R**	S	S	S	S
ESE	SE30, SE41, SE67	S	S	S	S	S	S	S	S
	ESE24	**R**	S	**R**	S	S	S	S	S
	ESE32	**R**	S	S	S	S	S	S	S
ESO	SO8, SO10, SO23, SO38, SO47, SO65	S	S	S	S	S	S	S	S
	SO24	S	S	S	**R**	S	S	S	S
	SO53	S	S	**R**	S	S	S	S	S
	SO56	S	S	**R**	S	S	S	S	S
ECE	CE2	S	S	S	S	S	S	S	S
	CE3	S	S	**R**	S	S	S	S	S
	CE4	S	S	**R**	S	S	S	S	S
	CE6	S	S	S	**R**	S	S	S	S
	CE10	**R**	S	**R**	S	S	S	S	S
	CE15	**R**	S	**R**	S	S	S	S	S
	CE5	**R**	**R**	**R**	S	S	**R**	**R**	S
ECO	CO38	S	S	S	S	S	S	S	S
	CO19	**R**	S	S	**R**	S	S	S	S
HNS	KN21, KN28, KN48	S	S	S	S	S	S	S	S
	KN3	S	S	S	**R**	S	S	S	S
	KN7	S	S	S	**R**	S	S	S	S
	KN12	S	S	S	**R**	S	S	S	S
	KN16	S	S	S	**R**	S	S	S	S
HS	KS8	S	S	S	**R**	S	S	S	S
	KS1	S	**R**	**R**	S	S	S	S	S
	KS2	S	**R**	**R**	S	S	S	S	S
	KS42	S	**R**	S	S	S	**R**	S	S
	KS13	S	**R**	**R**	**R**	S	S	S	S

	ZK39, ZK42, ZK50	S	S	S	S	S	S	S	S
	ZK5	S	S	**R**	S	S	S	S	S
	ZK15	S	S	**R**	S	S	S	S	S
	ZK6	S	S	S	**R**	S	S	S	S
	ZK10	S	S	S	**R**	S	S	S	S
	ZK11	S	S	S	**R**	S	S	S	S
	ZK12	S	S	S	**R**	S	S	S	S
HZK	ZK47	S	S	S	**R**	S	S	S	S
	ZK23	**R**	S	S	S	S	S	S	S
	ZK18	**R**	S	**R**	S	S	S	S	S
	ZK53	**R**	S	**R**	S	S	S	S	S
	ZK26	S	**R**	S	S	S	S	**R**	S
	ZK30	S	**R**	S	S	S	**R**	S	S
	ZK19	S	**R**	**R**	S	S	**R**	S	S
	ZK36	**R**	**R**	**R**	S	S	S	S	S

Table 3.13: *Lat. curvatus* antibiotic-resistance.

Fermented sausages	***Lat. curvatus* strains**	**Gen**	**Kan**	**Str**	**Tet**	**Ery**	**Clin**	**Chlor**	**Amp**
IAL	IAL6	S	S	S	S	S	S	S	S
ESA	SA38, SA53	S	S	S	S	S	S	S	S
ESB	SB8	S	S	S	S	S	S	S	S
ESE	SE3, SE19	S	S	S	S	S	S	S	S
	SO59	S	S	S	S	S	S	S	S
	SO6	S	S	S	**R**	S	S	S	S
	SO13	S	S	S	**R**	S	S	S	S
	SO14	S	S	S	**R**	S	S	S	S
ESO	SO25	S	S	S	**R**	S	S	S	S
	SO16	S	S	**R**	S	S	S	S	S
	SO61	S	S	**R**	S	S	S	S	S
	SO19	S	S	**R**	S	S	S	S	**R**
	SO52	S	S	**R**	**R**	**R**	S	S	S
ECB	CB11, CB12	S	S	S	S	S	S	S	S
	CE32, CE35, CE37, CE42, CE46, CE51, CE52, CE54, CE57	S	S	S	S	S	S	S	S
	CE16	S	S	S	S	S	S	**R**	S
ECE	CE25	S	S	S	S	S	S	**R**	S
	CE53	S	S	S	S	S	S	**R**	S
	CE27	S	S	S	**R**	S	S	S	S
	CE40	S	S	**R**	S	S	S	S	S
ECO	CO46	S	S	S	S	S	S	S	S
	KN20, KN55	S	S	S	S	S	S	S	S
HNS	KN1	S	S	**R**	S	S	S	S	S
	KN18	**R**	S	**R**	S	S	S	S	**R**
	ZK3	**R**	**R**	**R**	S	S	S	S	S
HZK	ZK32	**R**	**R**	**R**	S	S	S	S	S
	ZK43	**R**	S	**R**	S	S	**R**	S	S
	ZK49	**R**	**R**	**R**	S	**R**	**R**	S	S

For this reason, in Table 3.14 only the data regarding Spanish and Croatian samples are reported. Considering *Lat. sakei*, the most representative isolated species, 45 strains out of 71 showed resistance to at least one antibiotic. A total of 28 strains presented only 1 resistance, while a discrete number of multi-resistant strains was found. In particular, 11 and 5 strains showed resistance against 2 and 3 tested antibiotics, respectively, while only 1 strain (CE5) isolated from ECE Spanish product, presented 5 antibiotic-resistances. For what concerns the antibiotic classes, these results demonstrated that there was a high resistance to aminoglycosides, especially for streptomycin characterised by 25 resistant strains, followed by gentamicin (13 resistant strains) and kanamycin (11 resistant strains). Another antibiotic against which a high strains resistance was demonstrated was tetracycline, with 15 resistant strains (Table 3.14).

The genotypic analysis on *Lat. sakei* showed the presence of two encoding genes for tetracycline resistance: *tetS* and *tetM*. SB57 and KN3 strains were characterised by both these genes, while KN7 and CO19 presented only *tetS* and *tetM*, respectively. No strains presented resistant genes for erythromycin.

Regarding *Lat. curvatus*, 19 strains out of 39 were recognised as resistant (Table 3.14). In particular, 12 strains showed one resistance, while multiple resistances (from 2 to 5 antibiotics) were detected into 7 strains, mainly belonging to Croatian products (SO19, SO52, KN18, ZK3, ZK32, ZK43 and ZK49). Moreover, also in this case, the data showed that the principal resistance was against aminoglycosides antibiotic class: 11 strains presented resistance to streptomycin, followed by gentamicin and kanamycin, with 5 and 3 resistant strains, respectively. Among the other antibiotics, tetracycline determined the highest number of resistant strains. Furthermore, the presence of the *ermB* encoding gene for erythromycin resistance was observed into two *Lat. curvatus* strains (SO52 and ZK49), confirming the result achieved with the micro dilution method.

Table 3.14: Antibiotic-resistances distribution regarding *Lat. sakei* and *Lat. curvatus* strains belonging to Spanish and Croatian samples.

Countries	Fermented sausages	Species	n° strains	n° resistant strains*	Antibiotic-resistances distribution							
					Gen	Kan	Str	Tet	Ery	Clin	Chlor	Amp
Spain	ESA	*Lat. sakei*	9	7	3	2	6	-	-	-	-	-
		Lat. curvatus	2	-	-	-	-	-	-	-	-	-
	ESB	*Lat. sakei*	10	3	-	-	2	1	-	-	-	-
		Lat. curvatus	1	-	-	-	-	-	-	-	-	-
	ESE	*Lat. sakei*	5	2	2	-	1	-	-	-	-	-
		Lat. curvatus	2	-	-	-	-	-	-	-	-	-
	ESO	*Lat. sakei*	9	3	-	-	2	1	-	-	-	-
		Lat. curvatus	9	8	-	-	4	5	1	-	-	1
	ECB	*Lat. curvatus*	2	-	-	-	-	-	-	-	-	-
	ECE	*Lat. sakei*	7	6	3	1	5	1	-	1	1	-
		Lat. curvatus	14	5	-	-	1	1	-	-	3	-
	ECO	*Lat. sakei*	2	1	1	-	-	1	-	-	-	-
		Lat. curvatus	1	-	-	-	-	-	-	-	-	-
Croatia	HNS	*Lat. sakei*	7	4	-	-	-	4	-	-	-	-
		Lat. curvatus	4	2	1	-	2	-	-	-	-	1
	HS	*Lat. sakei*	5	5	-	4	3	2	-	1	-	-
	HZK	*Lat. sakei*	17	14	4	4	6	5	-	2	1	-
		Lat. curvatus	4	4	4	3	4	-	1	2	-	-
	TOT	*Lat. sakei*	**71**	**45**	**13**	**11**	**25**	**15**	**-**	**4**	**2**	**-**
		Lat. curvatus	**39**	**19**	**5**	**3**	**11**	**6**	**2**	**2**	**3**	**2**

***: resistant at least to one of the tested antibiotics**

Comparing the two LAB dominant species for antibiotic-resistance, streptomycin resulted the most spread resistance both in *Lat. sakei* and *Lat. curvatus* isolates, followed by tetracycline. Otherwise, differently from *Lat. curvatus*, *Lat. sakei* isolates presented no resistance to ampicillin and erythromycin (Table 3.14).

Among resistant strains, streptomycin (55.6% and 57.9% for *Lat. sakei* and *Lat. curvatus*, respectively), tetracycline (33.3% and 31.6%), gentamicin (28.9% and 26.3%) and kanamycin (24.4% and 15.8%) resistances were the most detected (Figure 3.10). A lower number of strains showed resistance to other antibiotics, in particular 8.9% of *Lat. sakei* was resistant to clindamycin and 4.4% to chloramphenicol, while 15.8% of *Lat. curvatus* was resistant to chloramphenicol and 10.5% to clindamycin, erythromycin and ampicillin (Figure 3.10).

Fermented sausages from Italy (IM1, IM2 and IAL), Slovenian samples (SN and SWO) and the Spanish ECB product were characterised by the presence of susceptible biotypes, with no resistances to any antibiotic tested during this screening (Table 3.12).

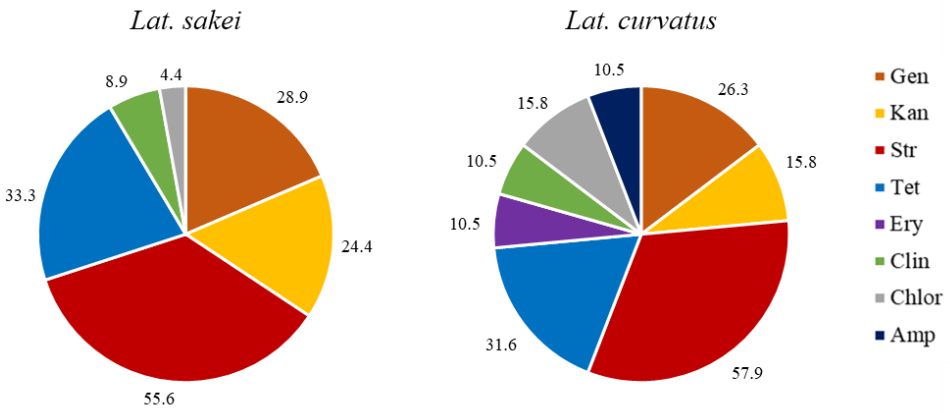


Figure 3.10: Relative abundance (%) of resistant biotypes for each species and antibiotics tested (% was calculated on the number of resistant strains).

- Biogenic amines production

Amino biogenic potential results, as well as for the antibiotic-resistance, demonstrated high variability among strains between those of the same species and, as already evidenced for antibiotic-resistance, also the decarboxylase activity was strongly linked to the geographic origin of the isolates. All the biotypes were screened for their possible production of tyramine (Tyr), histamine (Hist), putrescine (Put) and cadaverine (Cad).

No BA producers were detected among *Lat. sakei* strains, while a high number of *Lat. curvatus* (27 out of 40 strains) accumulated these compounds (Table 3.15).

The highest number of BA producing strains have been isolated from Spanish products, indicating an effect of raw materials, environmental conditions and processes in exerting a selective pressure on microbial communities and their metabolisms. Among the 27 decarboxylase-positive strains, 16 produced tyramine, 6 putrescine and only 1 histamine (CE27), while 4 strains were able to accumulate both tyramine and putrescine (SO14, SO59, CE16 and CE46).

Table 3.15: BA production by *Lat. curvatus*.

Fermented sausages	*Lat. curvatus* strains	Tyr*	Hist	Put	Cad
IAL	IAL6	+	-	-	-
ESA	SA38	+	-	-	-
	SA53	+	-	-	-
ESB	SB8	-	-	+	-
ESE	SE3	-	-	+	-
	SE19	-	-	+	-
ESO	SO6, SO19, SO25, SO52	-	-	-	-
	SO13	+	-	-	-
	SO16	+	-	-	-
	SO61	-	-	+	-
	SO14	+	-	+	-
	SO59	+	-	+	-
ECB	CB11	+	-	-	-
	CB12	+	-	-	-
ECE	CE25, CE40, CE53	-	-	-	-
	CE32	+	-	-	-
	CE35	+	-	-	-
	CE37	+	-	-	-
	CE42	+	-	-	-
	CE52	+	-	-	-
	CE54	+	-	-	-
	CE27	-	+	-	-
	CE51	-	-	+	-
	CE57	-	-	+	-
	CE16	+	-	+	-
	CE46	+	-	+	-
ECO	CO46	+	-	-	-
HNS	KN1, KN18, KN55	-	-	-	-
	KN20	+	-	-	-
HZK	ZK32, ZK43, ZK49	-	-	-	-
	ZK3	+	-	-	-
Total strains	**40 of which 27 positives**	**20**	**1**	**10**	**0**

*: Tyr = tyramine; Hist = histamine; Put = putrescine; Cad = cadaverine

It is interesting to note that 7 out of 27 amino biogenic strains presented one or more antibiotic-resistances, showing different traits that are related to their safety features. By combining the data collected for the antibiotic-resistance profile and the capability to produce BA compounds, it emerged that, among *Lat. curvatus*, only KN55 strain was safe and therefore suitable for possible food industry applications (Table 3.16).

Table 3.16: Summary of the results obtained through *Lat. curvatus* biotypes safety investigation: the presence of (S) or (R) indicates an antibiotic-sensitive or antibiotic-resistant strain, respectively. The amino biogenic potential is also reported (+ or -).

Fermented sausages	*Lat. curvatus* strains	Antibiotic-resistance behaviour	BA producer
IAL	IAL6	S	+
ESA	SA38, SA53	S	+
ESB	SB8	S	+
ESE	SE3, SE19	S	+
ESO	SO59	S	+
	SO6, SO19, SO25, SO52	**R**	-
	SO13, SO14, SO16, SO61	**R**	+
ECB	CB11, CB12	S	+
ECE	CE32, CE35, CE37, CE42, CE46, CE51, CE52, CE54, CE57	S	+
	CE25, CE40, CE53	**R**	-
	CE16, CE27	**R**	+
ECO	CO46	S	+
HNS	**KN55**	**S**	**-**
	KN20	S	+
HZK	KN1, KN18	**R**	-
	ZK32, ZK43, ZK49	**R**	-
	ZK3	**R**	+

Apart from enterococci, *Lat. curvatus* is considered the main tyramine producer among LAB in fermented sausages, while *Lat. sakei* is usually described as non-amino biogenic (Barbieri et al., 2019; Holck et al., 2017). Ladero et al. (2015) described the capability of *Lat. curvatus* strains to produce both tyramine and putrescine. The latter is mainly accumulated in LAB though agmatine deiminase (AgDI) pathway, rather than ornithine decarboxylase (ODC), common in Gram-negative bacteria (Romano et al., 2012). Moreover, the decarboxylase potential has been demonstrated to be strain dependent (Barbieri et al., 2019; Freiding et al., 2011).

Spontaneously fermented sausages used as source of isolation of these LAB presented a BA concentration ranging from about 100 mg/kg to more than 1000 mg/kg, including tyramine, putrescine and cadaverine. Interestingly, in these products *Lat. curvatus* strains characterised by decarboxylase potential have been isolated. Noteworthy, in ECE sample, in which *Lat. curvatus* CE27 (the only producer of histamine) was isolated, histamine was present at a concentration of 170 mg/kg (Table 3.6).

Finally, based on the results obtained from the screening for the safety aspect of the 130 tested LAB strains (90 *Lat. sakei* and 40 *Lat. curvatus*), 45 *Lat. sakei* and 1 *Lat. curvatus* strains showed no antibiotic-resistance and no capability to produce BA (Figure 3.11). These 46 strains

were selected for further analyses in order to evaluate their antimicrobial activity and their technological properties.

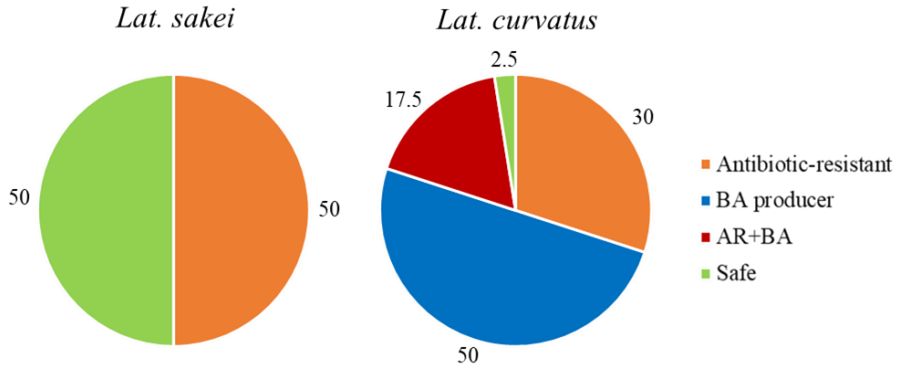


Figure 3.11: Percentage of tested LAB strains as far as their safety aspect: strains considered safe, antibiotic-resistant strains, BA producers or the combination of these two aspects (AR+BA).

3.2.4 Conclusions

The 15 Mediterranean spontaneously fermented sausages analysed in the first part of this thesis (Chapter 3.1), with peculiar characteristics in terms of manufacturing and ripening conditions, demonstrated to be a good source of autochthonous LAB to be studied for their potential technological applications in the food industry. At species level, the identified biotypes did not show a consistent biodiversity, with only *Lat. sakei*, dominating over the rest of the species (59.6% of isolates), *Lat. curvatus* (26.5%) and few isolates identified as *Comp. alimentarius* (13.9%). A more consistent biodiversity could be described in terms of strain ecology, with the Spanish products being the richest for the number of biotypes, while Italian and Slovenian samples showed only a low number of strains, belonging mainly to *Lat. sakei* species.

The evaluation of the safety profile of these strains resulted in a high incidence of *Lat. sakei* (50%) and *Lat. curvatus* (47.5%) resistant to antibiotics. In addition, the safety assessment allowed to define a geographical clustering of resistant biotypes: strains isolated from Italian and Slovenian natural fermented sausages showed no antibiotic-resistances and a negligible production of BA; on the contrary, the highest number of antibiotic-resistant isolates were detected in Spanish and Croatian products, with a high prevalence of multi-antibiotic resistant strains. In fact, one *Lat. sakei* (CE5) and one *Lat. curvatus* (ZK49) characterised by the presence of five resistances against tested antibiotics were isolated from ECE and HZK samples, respectively. In general, the most observed antibiotic-resistances concerned streptomycin, tetracycline, gentamicin and kanamycin antibiotics.

This aspect arises a global concern linked to the safety of fermented meat products. The previous large use of antibiotics in the pig production chain has led to a change in the pig

microbiome and, consequently, in the diffusion of antibiotic resistant genes in the meat environment (Monger et al., 2021). The application of good manufacturing practices in the pork meat industry can help to control antibiotic resistant pathogen or spoilage species. On the contrary, in the case of technological species, such as LAB, the presence of resistant genes, can represent a difficult risk to be monitored for the consumer safety along the food chain.

Finally, amino biogenic potential appeared to be species related. In fact, no BA producers were detected among analysed *Lat. sakei* strains, while a high number of strains endowed with this feature was found among *Lat. curvatus*.

After the safety assessment, a total of 46 LAB strains (45 *Lat. sakei* and 1 *Lat. curvatus*) were classified as safe, without antibiotic-resistances and amino biogenic capacity. *Lat. sakei* demonstrated to be the most abundant species present in naturally fermented Mediterranean sausages, but also to be the species with the best safety features. These results were the starting point for the characterisation of candidates to be tested for their technological attributes and bio-protective activities. These strains could be proposed as autochthonous bioprotective and starter cultures with added value to be employed in the fresh and fermented meat productions.

Data published as:

Bassi, D.*, Milani, G., Belloso Daza, M.V., **Barbieri, F.**, Montanari, C., Lorenzini, S., Šimat, V., Gardini, F., Tabanelli, G. (2022). Taxonomical identification and safety characterisation of *Lactobacillaceae* from Mediterranean natural fermented sausages. *Foods*, 11, 2776. doi: 10.3390/foods11182776.

3.3 Functional and technological characterisation of LAB strains isolated from Mediterranean spontaneously fermented sausages

3.3.1 Introduction

Food-borne pathogens are one of the major cause of outbreaks and mortality worldwide, being one of the greatest health concerns for governments, which incur in greater access to the healthcare and medical expenses (Erdoğmuş et al., 2021; Faour-Klingbeil and Todd, 2020). Their persistence, growth and ability to create biofilms and possible toxin production highlighted their dangerous aspect (Kim et al., 2012a; Woraprayote et al., 2016).

Furthermore, consumers' demand for healthy and safe food products is constantly increasing during recent years (de Andrade et al., 2019).

Manufacturing of standardised and safe food products, but still characterised by their traditional and linked-Region organoleptic and nutritional properties represents a main node for food companies, which aim to find a strategy to achieve all the markets' requests (Gizaw, 2019). In this perspective, the use of autochthonous starter cultures can be a useful tool to achieve the production of traditional foods that are also safe from a health point of view. Moreover, indigenous starter cultures are known to positively affect taste, texture and colour of fermented foods (dos Santos Cruxen et al., 2019).

Among processed foods, meat and meat products constitute a significant challenge to the food industry regarding their safety. In fact, possible microbial contaminations of fresh and processed products by various pathogenic and spoilage microorganisms can occur, without being hindered only by change in setting of production phases (Lahiri et al., 2022). These concerns can be mitigated by the use of LAB, that can guide the fermentation process in order to produce safe food thanks to their positive properties, such as suitable technological characteristics and the ability to inhibit the growth of undesired microorganisms in food (Mathur et al., 2020; Raman et al., 2022). LAB are naturally present in raw materials or processed products and, due to their GRAS (Generally Recognised As Safe) status, are widely used in the meat industry as transformation and biocontrol agents (Castellano et al., 2017).

Antimicrobial activity of LAB strains is exerted by different metabolites produced during their growth and fermentation. These microorganisms are effective in inhibit the growth of a wide range of food-borne pathogens and spoilage agents, due to their production of several bio-active compounds: organic acids, diacetyl, acetoin, hydrogen peroxide and bacteriocins (da Costa et al., 2019).

Several studies documented that a wide range of LAB strains, which include *Lat. sakei*, *Ltp. plantarum* and *Lat. curvatus*, can be used as bio-preservative microorganisms in meat and meat products (Castellano et al., 2012; Jones et al., 2010; Li et al., 2016). Their inhibitory activity have been extensively studied to evaluate the effect against pathogenic bacteria or spoilage microorganisms' growth, when inoculated into foodstuffs (Castellano et al., 2010; Chaillou et al., 2014; Todorov et al., 2017).

Food industries are continuously searching for new autochthonous bacteria (LAB and Gram-positive catalase cocci), endowed with technological and antimicrobial features, that can be potentially used as starter cultures in fermented meat products (dos Santos Cruxen et al., 2019).

In this prospective, 45 *Lat. sakei* and 1 *Lat. curvatus* strains, isolated from spontaneously fermented sausages of the Mediterranean area and previously screened regarding their safety aspects (Chapter 3.2), were characterised for their ability to inhibit pathogen microorganisms through *in vitro* tests and in meat model. Moreover, the presence of genes related to the production of bacteriocins was assessed. Afterwards, the most promising strains were studied to determine their technological properties. Growth kinetics at different salt concentrations and at different incubation temperatures were studied.

3.3.2 Materials and methods

3.3.2.1 LAB strains selected to perform antimicrobial activity against *E. coli* and *List. innocua*

46 LAB strains (45 *Lat. sakei* and 1 *Lat. curvatus*), considered safe (Chapter 3.2), were tested against *E. coli* ATCC 25922 and *List. innocua* UC 8409, in order to assess their antimicrobial activity (Table 3.17). All bacteria were stored at -20°C in MRS broth and BHI broth, respectively, containing 20% of glycerol until the beginning of further analyses.

Table 3.17: Selected LAB strains assessed as safe, obtained from the 15 Mediterranean spontaneously fermented sausages used as source of LAB isolation.

Countries	Fermented sausages	LAB strains	Species
Italy	1M	1M8, 1M24, 1M51	
	2M	2M7, 2M9	
Slovenia	IAL	IAL8, IAL18, IAL38	
	SN	SN4, SN34, SN58, SN63, SN70	
	SWO	SWO10, SWO18, SWO29, SWO48, SWO60, SWO61	
Spain	ESA	SA21, SA49	
	ESB	SB2, SB7, SB14, SB24, SB53, SB60, SB67	***Lat. sakei***
	ESE	SE30, SE41, SE67	
	ESO	SO8, SO10, SO23, SO38, SO47, SO65	
	ECE	CE2	
	ECO	CO38	
Croatia	HNS	KN21, KN28, KN48	
	HZK	ZK39, ZK42, ZK50	
	HNS	KN55	***Lat. curvatus***

3.3.2.2 *In vitro* antimicrobial LAB strain activity to inhibit *E. coli* and *List. innocua*

The antimicrobial activity of selected LAB strains was evaluated through an agar spot test using *E. coli* ATCC 25922 and *List. innocua* UC 8409 strains as target microorganisms.

Briefly, after LAB strains incubation at 30°C for 24 h, 10 µl of these fresh cultures were spotted on MRS agar plates and incubated for 48 h at 30°C. Afterwards, plates were overlayed with 10 ml of BHI soft agar (0.7%) inoculated with 5 log cfu/ml of an overnight culture of target microorganisms. Positive antimicrobial activity was evidenced after 24 and 48 h at 37°C as a clear inhibition zone that affected the target microorganism's growth around the spot. After the incubation period, the inhibition halos diameter was measured and the results were expressed as + (diameter 0.5-2 cm), ++ (diameter 2.1-4 cm), +++ (diameter > 4 cm) or – in the case of no presence of halo around the spot. The analysis was performed in triplicate.

3.3.2.3 Antimicrobial LAB strains activity in meat model

Selected LAB strains were also tested against the same target microorganisms (*E. coli* ATCC 25922 and *List. innocua* UC 8409) to evaluate their antimicrobial activity in meat model, in order to simulate the real conditions of a food matrix on a lab-scale. In particular, agar-salami medium was used and its composition is reported in Table 3.18.

Table 3.18: Agar-salami meat model composition.

Composition	Quantity	Final concentration
Salami dough	60 g	
Water	12 ml	
Agar bacteriological 2%	180 ml	
Glucose 20%	6.3 ml	0.5%
2,3,5-Triphenyltetrazolium chloride 1%	2.5 ml	0.01% TTC
Final volume	250 ml	

To prepare this medium, 60 g of meat mixture was finely minced in a sterile condition pasteurised at 65°C for 30 min. At the end of the heat treatment, 12 ml of sterile water and 180 ml of 2% agar solution were added into the dough. The mixture was filtered using sterile gauze in order to remove particulate material. After filtration, 20% glucose and 1% TTC (2,3,5-Triphenyltetrazolium chloride) solutions were added. This latter compound allowed the red staining of microbial growth.

The inhibition tests were performed in square set plates of 25 wells (ThermoFisher Scientific) (Figure 3.12). On the bottom of the plates, 30 µl of overnight selected LAB cultures were spotted separately at a final concentration of 6 log cfu/ml. Subsequently, the agar-salami medium was poured at a temperature of 50°C in all 25 wells of the plate (4 ml in each well), homogenising the inoculum with the mixture. Plates were incubated at 30°C for 24 h to allow LAB growth. Then, 30 µl of ten-fold dilutions of a 6 log cfu/ml culture of target microorganism were spotted in each well and plates were incubated at 37°C for 24 h. The positive control was performed without the addition of LAB strains. In addition, at the end of incubation time, the pH value in each well was measured and indicated as the mean value of eight grown wells. The inhibition effect on the target microorganisms was expressed as a logarithmic concentration reduction compared to the positive control.

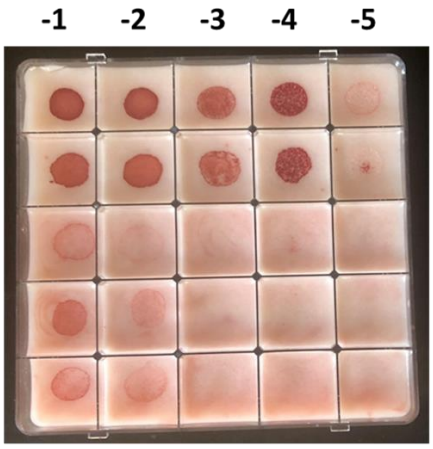


Figure 3.12: Square set plates of 25 wells used to perform these antimicrobial analyses, an example.

3.3.2.4 Gene-specific PCR analysis for the detection of bacteriocin encoding genes

The presence of genes related to the production of bacteriocins was also investigated in each selected LAB strain. In particular, the presence of bacteriocin encoding genes for sakacin P (*sppA*), sakacin Q (*sppQ*), sakacin T (*sakT*), sakacin X (*sakX*), sakacin G (*sakG*) and curvacin A (*sapA*) were evaluated.

LAB strains DNA was extracted by using the NucleoSpin® Tissue (Macherey-Nagel GmbH & Co., Düren, Germany) according to the manufacturer's instructions. Primers and PCR conditions used were those reported by Fontana et al. (2015), Dortu et al. (2008) and Macwana and Muriana (2012). The amplified products were separated in 1.5% agarose gel and visualised by Sybr-Safe staining. Positive controls for each encoding genes were also included and to perform this *Lat. curvatus* M05, *Lat. curvatus* 705 and *Lat. curvatus* R212 were used.

3.3.2.5 Selected strains employed for technological characterisation

Among strains considered safe, based on results obtained from their antimicrobial activity characterisation, the most promising LAB strains were selected and evaluated for their technological properties. In particular, due to their better bio-protective performances, 19 *Lat. sakei* and 1 *Lat. curvatus* were chosen to perform further technological analyses (Table 3.19).

Table 3.19: Selected strains used for their technological characterisation.

Countries	Fermented sausages	LAB strains	Species
Italy	IM1	1M24	*Lat. sakei*
	IM2	2M7	
		2M9	
	IAL	IAL8	
Slovenia	SN	SN34	
		SN58	
	SWO	SWO10	
		SWO61	
Spain	ESB	SB2	
		SB14	
	ESE	SE30	
	ESO	SO8	
		SO23	
		SO65	
	ECE	CE2	
	ECO	CO38	
Croatia	HNS	KN48	
	HZK	ZK39	
		ZK42	
	HNS	KN55	*Lat. curvatus*

3.3.2.6 Study of LAB growth performances in presence of different salt concentrations and at different incubation temperature

Selected LAB strains were evaluated for their growth performances in relation of different salt concentrations (0%, 2.5% and 5% NaCl) and at different incubation temperatures (10°C, 20°C and 30°C). They were pre-cultivated in MRS broth (Oxoid) for 24 h at 30°C and then inoculated at a final concentration of 5 log cfu/ml into different media chosen for the analyses. In particular, samples characterised by different salt concentrations were incubated at 20°C, while strains incubated at different temperatures were inoculated in unmodified MRS. During incubation, their growth was monitored through the variation of optical density at 600 nm (OD_{600}), measured with an UV-VIS spectrophotometer 6705 UV-Vis (Jenway, Stone, UK). Moreover, pH values were also detected by pH-meter Basic 20 (Crison Instruments) and the acidification potential was expressed as pH decrease with respect to the initial value (about 6).

Collected data were elaborated by using predictive microbiology models. In this context, Gompertz equation, as modified by Zwietering et al. (1990), was used to model them with Statistica 8.0 software (StatSoft Inc.):

$$y = Ae^{-e^{\left[\left(\frac{\mu_{max}e}{A}\right)(\lambda - t)+1\right]}}$$

when y is the OD_{600} measured at time t, *A* represent the maximum OD_{600} value reached, μ_{max} is the maximum OD_{600} increase rate in exponential phase and λ is the lag phase. In the case of pH elaboration data, a constant k was added into equation to specify the starter value of this parameter.

3.3.2.7 Aroma profile analysis of LAB strains in a real system

Volatile organic compounds (VOCs) production of selected LAB belonging to *Lat. sakei* and *Lat. curvatus* species were evaluated through their inoculum in meat batter to simulate a meat fermentation. The pork meat used, without nitrate/nitrite and spices, was added with 2.5% NaCl and 0.2% glucose. The strains were pre-cultivated in MRS broth at 30°C for 24 h. 50 g of meat batter were inoculated with each strain, separately, at an initial concentration about 7 log cfu/g. Afterwards, 3 g of sample were placed in 10 ml sterilised vials sealed with PTFE/silicon septa. The obtained samples were incubated at 20°C for 72 h, then stored at -20°C until analysis. Aroma profile of each sample was evaluated through a gas-chromatography-mass spectrometry coupled with solid-phase microextraction (SPME-GC-MS), following the method showed into Paragraph 3.1.2.6.

3.3.2.8 Statistical analysis

Parameters obtained with Gompertz equation modelling were elaborated through statistical software R (R Core Team, 2020). The analyses were performed by using “boxplot” function.

3.3.3 Results and discussion

3.3.3.1 Antimicrobial activity of selected LAB strains against *E. coli* and *List. innocua*: *in vitro* tests

Based on the previously analyses regarding safety aspects of isolated LAB strains (Chapter 3.2), the evaluation of their functional properties was performed onto strains considered safe (Figure 3.11). In particular, the antimicrobial activity of 46 selected LAB strains (45 *Lat. sakei* and 1 *Lat. curvatus*) was evaluated against *E. coli* ATCC 25922 and *List. innocua* UC 8409, as representative of pathogens strains of *E. coli* and *List. monocytogenes*.

As reported in Table 3.20, all tested strains were able to inhibit the growth of both target microorganisms, even if at different levels depending on the specific strain. In particular, *E. coli* ATCC 25922 represented the most sensible microorganism to LAB inhibition. In fact, among *Lat. sakei*, 7 strains, in particular belonging to Croatian products, produced an inhibition zone higher than 4 cm, while the most number (31 strains) showed a halo ranging from 2.1 and 3.2 cm. On the other hand, *List. innocua* UC 8409 was inhibited less severely, with 33 strains that created an inhibition zone between 1.6 and 2 cm and only 4 strains that affected the target microorganism growth with a halo between 2.1 and 3.2 cm. Strains that strongly inhibited *List. innocua* were the same that influenced *E. coli*, with an inhibition halo > 4 cm. Finally, the only *Lat. curvatus* strain tested showed a promising inhibition performance against both tested microorganisms, with an inhibition zone > 4 cm.

Table 3.20: Inhibition halo of LAB strains against *E. coli* ATCC 25922 and *List. innocua* UC 8409. Inhibition zones: (+++) = > 4 cm; (++) = 2.1-4 cm; (+) = 0.5-2 cm; (-) no halo.

Fermented sausages	***Lat. sakei* strains**	***E. coli* ATCC 25922**	***List. innocua* UC 8409**
IM1	1M8, 1M24, 1M51	(++)	(+)
IM2	2M7, 2M9	(++)	(+)
IAL	IAL8, IAL18	(+)	(+)
	IAL38	(+)	(++)
SN	SN4, SN34, SN58, SN63, SN70	(++)	(+)
SWO	SWO18, SWO29, SWO60, SWO61	(++)	(+)
	SWO48	(++)	(++)
ESA	SA21, SA49	(++)	(+)
ESB	SB14, SB24, SB53, SB60, SB67	(++)	(+)
	SB2	(++)	(++)
	SB7	(+++)	(+++)
ESE	SE30, SE41, SE67	(++)	(+)
ESO	SO47, SO65	(+)	(+)
	SO8, SO10, SO38	(++)	(+)
	SO23	(++)	(++)
ECE	CE2	(+)	(+)
ECO	CO38	(++)	(+)
HNS	KN21, KN28, KN48	(+++)	(+++)
HZK	ZK39, ZK42, ZK50	(+++)	(+++)
Fermented sausages	***Lat. sakei* strains**	***E. coli* ATCC 25922**	***List. innocua* UC 8409**
HNS	KN55	(+++)	(+++)

3.3.3.2 Antimicrobial LAB strains activity in meat model (agar-salami medium)

The antimicrobial activity of LAB strains against tested target microorganisms was also evaluated in a meat model, defined by agar-salami medium, that has the same nutritional and physico-chemical characteristics of real products. It was possible to determine the inhibitory activity through the comparison between the positive control, characterised by the growth of *E. coli* ATCC 25922 or *List. innocua* UC 8409 without the addition of LAB strains, and samples obtained with the presence of both target microorganism and LAB culture. The results were expressed as logarithmic cycles reduction compared to the positive control (Table 3.21).

All tested strains were able to inhibit the growth of target microorganisms with different severity, except for the strain IAL18 which did not cause any reduction of *List. innocua*. Moreover, data confirmed a greater sensitivity trend in *E. coli* to LAB strains inhibition effect with respect to *List. innocua*. In fact, 12 *Lat. sakei* strains reduced by at least 4 log cycles *E. coli* growth in the meat model, while only 3 strains isolated from HNS Croatian product (KN21, KN28 and KN48) inhibited this target microorganism of about 5 log cycles. Regarding *List. innocua*, 8 *Lat. sakei*

strains were able to reduce 4 log cycles of this target microorganism and only KN48 was able to achieve 5 log cycles *Listeria* reduction.

It is well known that acidity caused by the organic acid produced during LAB strains growth could affect the growth of microorganisms, due to the formation of a harsh environment. For this reason, also pH value at the end of incubation was monitored. In the majority of the most acidified samples, the values of log cycles reduction were greater with respect to less acidified samples. It was also possible to notice that the strains which showed the best inhibitory performances against *E. coli*, were the ones which mostly reduced *List. innocua*.

The higher resistance of *Listeria* to acidic environments was reported also by Wang et al. (2015), demonstrating that it was necessary an increased time of contact between of *List. monocytogenes* and lactic acid to achieve its inactivation, with respect to *Salmonella* spp. and *E. coli*.

Table 3.21: Log cycles reduction of target microorganisms as a consequence of antimicrobial activity of selected LAB strains. pH values measured at the end of the analyses were also reported.

		Target microorganisms log cycles reduction (log cfu/ml)		**pH value**	
Fermented sausages	***Lat. sakei* strains**	***E. coli* ATCC 25922**	***List. innocua* UC 8409**	***E. coli* ATCC25922**	***List. innocua* UC 8409**
IM1	1M8	1	1	4.16	4.15
	1M24	4	4	3.71	3.71
	1M51	4	3	3.63	3.63
IM2	2M7	3	2	3.94	3.96
	2M9	4	4	3.72	3.68
IAL	IAL8	3	2	4.07	4.10
	IAL18	1	-	4.32	4.08
	IAL38	2	3	3.69	3.65
SN	SN4	3	1	4.36	4.36
	SN34	4	4	3.72	3.70
	SN58	4	4	3.89	3.85
	SN63	2	1	3.89	3.94
	SN70	2	1	4.03	3.99
SWO	SWO10	4	4	3.65	3.70
	SWO18	2	1	4.11	4.11
	SWO29	3	2	3.75	3.74
	SWO48	3	3	3.70	3.68
	SWO60	2	2	3.91	3.90
	SWO61	3	2	3.84	3.94
ESA	SA21	4	3	3.85	3.85
	SA49	3	3	3.79	3.82
ESB	SB2	4	4	3.83	3.83
	SB7	2	1	4.03	4.07
	SB14	4	4	3.69	3.70
	SB24	3	3	3.82	3.85

	SB53	1	1	3.98	4.19
	SB60	3	2	3.97	4.17
	SB67	4	2	3.95	4.06
	SE30	3	2	3.87	3.85
ESE	SE41	2	1	3.97	4.03
	SE67	2	3	4.05	4.01
	SO8	2	3	4.04	4.00
	SO10	2	3	4.04	4.04
ESO	SO23	2	3	4.01	3.98
	SO38	1	2	3.90	3.93
	SO47	3	2	3.93	3.92
	SO65	3	3	3.86	3.89
ECE	CE2	3	1	3.87	3.91
ECO	CO38	4	1	3.64	3.83
	KN21	5	4	3.86	3.84
HNS	KN28	5	3	3.83	3.89
	KN48	5	5	3.77	3.78
	ZK39	1	3	4.29	4.13
HZK	ZK42	4	3	3.91	4.04
	ZK50	2	3	4.09	4.02
Fermented sausages	***Lat. curvatus* strains**	***E. coli* ATCC 25922**	***List. innocua* UC 8409**	***E. coli* ATCC25922**	***List. innocua* UC 8409**
HNS	KN55	2	3	4.12	4.14

3.3.3.3 Detection of the presence of bacteriocins encoding genes

The presence of bacteriocins encoding genes were investigated in all the strains screened for their antimicrobial activity. In particular, sakacin P (*sppA*), sakacin Q (*sppQ*), sakacin T (*sakT*), sakacin X (*sakX*), sakacin G (*sakG*) and curvacin A (*sapA*) were searched (Table 3.22).

24 strains of *Lat. sakei* were characterised by the presence of at least one bacteriocin, mainly for the production of sakacin X, followed by sakacin P. Among these, in 6 strains (2M7, SN58, SA21, SB2, SO65 and ZK50) 2 different bacteriocins encoding genes were detected, while 2 strains (SO8 and CO38) isolated from two different Spanish products were characterised by the presence of 3 bacteriocins encoding genes. The most interesting strains (ZK39 and ZK42), deriving from Croatian smoked fermented sausages, possessed 4 different genes encoding for bacteriocins. These data confirmed the results obtained in the previously analyses, in which these strains showed the highest antimicrobial activity against target microorganisms *in vitro* (Table 3.19) and a good potential in meat model (Table 3.21). Finally, also *Lat. curvatus* KN55 was characterised by 2 different encoding genes.

Table 3.22: Bacteriocin encoding genes found in *Lat. sakei* and *Lat. curvatus* strains.

Fermented sausages	***Lat. sakei* strains**	***sap*A**	***spp*A**	***spp*Q**	***sak*G**	***sak*T**	***sak*X**
IM1	1M-24						x
	1M8, 1M51						
IM2	2M7				x		x
	2M9						
IAL	IAL8, IAL18, IAL38						
SN	SN58		x				x
	SN34						x
	SN4, SN63, SN70						
SWO	SWO29, SWO61						x
	SWO10, SWO18, SWO48, SWO60						
ESA	SA21		x				x
	SA49						
ESB	SB2		x				x
	SB7		x				
	SB24, SB60, SB67						x
	SB14, SB53						
ESE	SE30, SE67						x
	SE41						
ESO	SO8		x	x			x
	SO65			x			x
	SO47						x
	SO10, SO23, SO38						
ECE	CE2						
ECO	CO38	x	x				x
HNS	KN21, KN28, KN48		x				
HZK	ZK39		x	x		x	x
	ZK42	x	x		x		x
	ZK50		x	x			
Fermented sausages	***Lat. curvatus* strain**	***sap*A**	***spp*A**	***spp*Q**	***sak*G**	***sak*T**	***sak*X**
HNS	KN55	x					x

3.3.3.4 Growth and acidification performances of selected LAB strains at different condition

The growth performances of the 20 selected strains were analysed in MRS broth, in which each strain was inoculate at an initial concentration about 5 log cfu/ml. This analysis permitted to compare the different growth parameters and to highlight the strains characterised by the best performances in stressful conditions. In particular, the presence of different salt concentration (0%, 2.5% and 5%, at 20°C of incubation) and incubation temperature (10°C, 20°C, 30°C, in absence of salt) were assessed and reported as optical density measured at 600 nm (OD_{600}). Data were modelled with the Gompertz equation and estimated parameters are reported in Table 3.23 and 3.24, while the resulting curves were represented in Figure 3.13 and 3.14.

Table 3.23: Gompertz parameters (A, μ_{max} and λ) about *Lat. sakei* and *Lat. curvatus* strains growth at 20°C in presence of different salt concentrations. Mean values, standard deviation (SD) and variability coefficient (CV) are also reported.

	OD_{600} 0%			OD_{600} 2.5%			OD_{600} 5%		
Strains	***A***	μ_{max}	λ	***A***	μ_{max}	λ	***A***	μ_{max}	λ
1M24	1.98	0.180	13.09	1.81	0.155	16.02	1.69	0.044	20.93
2M7	1.89	0.153	11.26	1.83	0.094	14.14	1.53	0.045	26.13
2M9	1.89	0.221	16.18	1.74	0.141	18.73	1.45	0.059	33.13
IAL8	1.84	0.161	14.63	1.83	0.130	16.06	1.62	0.055	22.91
SN34	1.68	0.247	16.81	1.50	0.137	18.84	1.28	0.091	37.10
SN58	1.91	0.174	12.38	1.69	0.090	14.07	1.16	0.038	21.53
SWO10	1.65	0.239	16.03	1.57	0.139	17.95	1.52	0.054	26.71
SWO61	1.98	0.114	7.14	1.96	0.094	10.06	1.72	0.058	20.76
SB2	1.69	0.146	13.40	1.64	0.104	19.09	1.63	0.033	29.01
SB14	1.98	0.110	8.73	1.87	0.091	14.02	1.61	0.045	24.67
SE30	1.95	0.217	15.58	1.69	0.135	18.94	1.48	0.047	31.31
SO8	1.97	0.227	13.99	1.80	0.142	15.76	1.46	0.043	28.32
SO23	1.82	0.174	16.94	1.69	0.096	18.05	1.45	0.043	27.12
SO65	1.96	0.160	10.04	1.91	0.111	12.77	1.64	0.060	21.13
CE2	1.96	0.122	8.68	1.88	0.106	13.61	1.59	0.048	19.30
CO38	1.99	0.086	17.82	1.96	0.068	16.88	1.81	0.036	18.49
KN48	1.99	0.143	10.30	1.86	0.097	12.53	1.61	0.037	17.11
ZK39	1.99	0.144	17.64	1.91	0.082	17.54	1.96	0.037	24.03
ZK42	1.95	0.132	12.64	1.79	0.087	15.69	1.53	0.033	21.90
KN55	2.06	0.227	13.56	1.98	0.146	15.72	1.64	0.056	25.13
Mean value	1.91	0.169	13.34	1.80	0.112	15.82	1.57	0.048	24.83
SD	0.11	0.047	3.19	0.13	0.026	2.50	0.17	0.013	5.14
CV (%)	6.02	27.87	23.91	7.37	22.92	15.82	11.06	27.74	20.68

As expected, the estimated growth parameters of tested strains were affected by the salt percentage, that resulted in a decrease in A and μ_{max} values and an increase in lag phase time with the increase in its concentration (Table 3.23). Regarding samples characterised by 0% and 2.5% of salt, strains presented a similar behaviour, reaching a maximum OD_{600} mean value of 1.91 and 1.80 and λ of 13.34 and 15.82 h, respectively, while μ_{max} parameter was characterised by a significative reduction up to 0.112 OD_{600}/h.

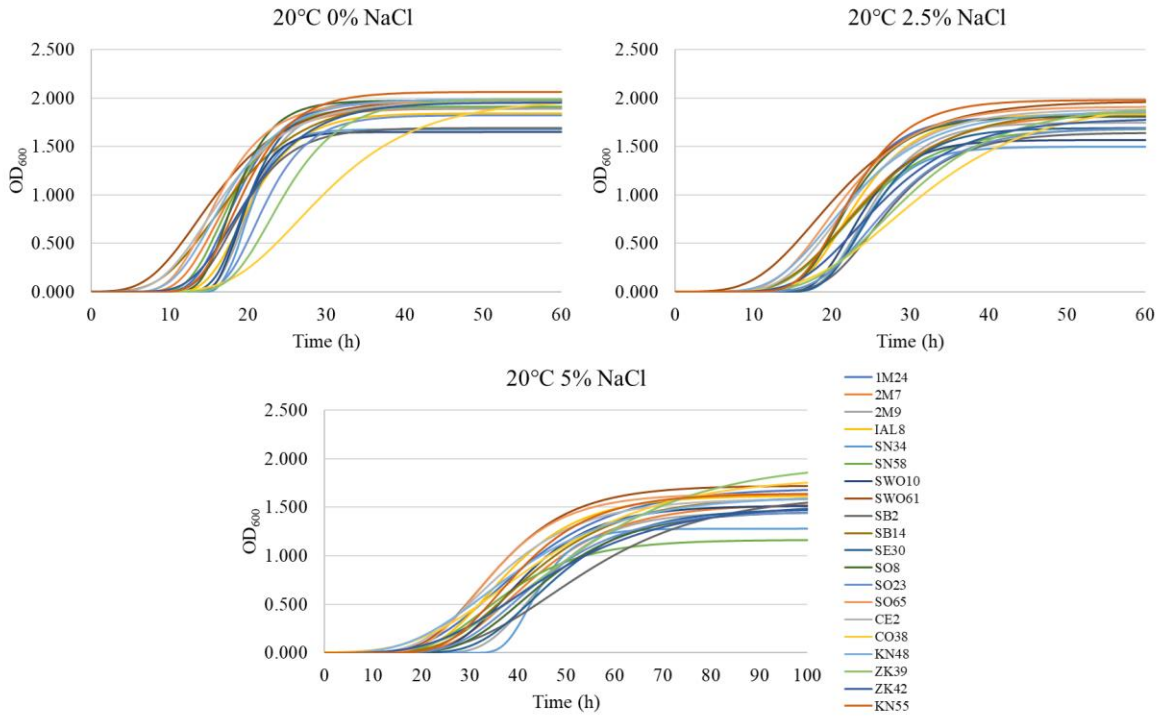


Figure 3.13: Growth curves at different salt concentrations (0%, 2.5% and 5%) obtained by using the estimated parameters of Gompertz equation.

The best performances were observed when KN55, the only *Lat. curvatus* strain tested, was inoculated (Figure 3.13). This strain reached the maximum *A* value at the end of the monitored growth, with the highest OD_{600} values of 2.06 and 1.98 when tested in absence of salt or with the medium concentration, respectively. The lower *A* parameter was achieved by *Lat. sakei* strains SN34, SWO10 and SB2. In the absence of salt, they were characterised by values ranged between 1.65 and 1.69 OD_{600}, significantly lower than the others (Table 3.23). In samples with 2.5% of added salt, this behaviour was observed again for these three strains (from 1.50 to 1.64 OD_{600}), but also SN54, SE30 and SO23 showed a low maximum OD_{600} value (1.69). Moreover, another good strain was represented by SWO61, characterised also by the shorter lag phase time (7.14 h). On the other hand, CO38 represents the worst strain at these growing conditions (Figure 3.13), not only due to the high λ measured, but also because it was affected by the slower OD_{600} increase rate in exponential phase (0.086 and 0.068 OD_{600}/h, respectively), even if at the end of the growth was able to reach an *A* value of about 1.98 OD_{600}, comparable to strains with higher values of the same parameter (Table 3.23). The μ_{max} parameter was characterised by a marked variability, with values ranged from 0.247 OD_{600}/h (SN34) and 0.086 OD_{600}/h (CO38) in absence of salt, described by a variability coefficient (CV) of 27.87%. At 2.5% of salt, this increase rate was slower and ranged from 0.146 OD_{600}/h (KN55) and 0.068 OD_{600}/h (CO38).

A greater variability was observed when the highest salt concentration (5%) was tested. In fact, the consequent decrease in the maximum OD_{600} measured (mean value 1.57 OD_{600}) and in the μ_{max} parameter (mean value 0.048 OD_{600}/h) and increase in lag phase time (mean value 24.83 h) were described by a CV of 11.06%, 27.74% and 20.68%, respectively (Table 3.23).

In this case, the maximum OD_{600} was reached by ZK39 strain (1.96 OD_{600}), even if its grow rate was not the faster (0.037 OD_{600}/h), followed by CO38 (1.81 OD_{600}) and SWO61 (1.72 OD_{600}). CO38 strain was characterised also by a small lag phase (18.49 h), together with KN48, that was described by the shorter one (17.11 h). The worst strain was represented by SN34, due to the higher lag phase time (37.10 h) and a low *A* value reached (1.28 OD_{600}). Moreover, 2M9 and SE30 strains were characterised by high λ values (33.13 h and 31.31 h, respectively), while the lower maximum OD_{600} observed was reached by SN58 (1.16 OD_{600}).

These results underlined the great variability that can characterise strains belonging to the same species. Montanari et al. (2018b) have already reported a different behaviour in the growth performances of *Lat. sakei* strains in stressful conditions.

In general, the results collected at different incubation temperature (10°C, 20°C and 30°C) reflected the behaviour observed in the condition of the different salt concentrations tested (0%, 2.5% and 5%), with a reduction in the growth performances in the most stressful condition. In particular, with the decrease of temperature up to 10°C the growth performances were worse, with an increase in the lag phase time and a decrease in the maximum *A* and μ_{max} values (Table 3.24). Moreover, the reduction in the incubation temperature determined a greater variability in data collected among the strains. These results were characterised by a mean value of *A* of about 1.90 OD_{600} both at 30°C and 20°C of incubation, while decrease up to 1.79 OD_{600} at 10°C. However, μ_{max} and λ parameters were characterised by a greater difference among the tested conditions. In fact, in the first case, at 30°C the μ_{max} mean value was 0.246 OD_{600}/h, that was halved at 20°C (0.169 OD_{600}/h) and drastically decreased at lower temperature of incubation, reaching value of 0.032 OD_{600}/h. Regarding lag phase, this parameter was affected by a 10-fold increase when samples were incubated at 10°C (54.18 h), whit respect to 30°C of incubation (6.76 h).

Table 3.24: Gompertz parameters (A, μ_{max} and λ) about *Lat. sakei* and *Lat. curvatus* strains growth at different incubation temperatures. Mean values, standard deviation (SD) and variability coefficient (CV) are also reported.

	OD_{600} 10°C			OD_{600} 20°C			OD_{600} 30°C		
Strains	***A***	μ_{max}	λ	***A***	μ_{max}	λ	***A***	μ_{max}	λ
1M24	1.83	0.039	52.12	1.98	0.180	13.09	1.91	0.278	5.30
2M7	1.86	0.031	56.33	1.89	0.153	11.26	1.93	0.178	7.41
2M9	1.92	0.054	33.81	1.89	0.221	16.18	1.90	0.282	7.45
IAL8	1.70	0.034	70.42	1.84	0.161	14.63	1.83	0.235	7.17
SN34	1.79	0.042	60.10	1.68	0.247	16.81	1.63	0.303	7.78
SN58	1.90	0.037	40.32	1.91	0.174	12.38	2.03	0.271	3.58
SWO10	1.58	0.070	34.00	1.65	0.239	16.03	1.68	0.302	7.98
SWO61	1.72	0.025	46.55	1.98	0.114	7.14	1.87	0.313	6.21
SB2	1.50	0.013	64.20	1.69	0.146	13.40	1.71	0.262	9.40
SB14	1.99	0.016	41.01	1.98	0.110	8.73	1.91	0.157	5.96
SE30	2.04	0.041	49.38	1.95	0.217	15.58	1.99	0.265	7.77
SO8	1.63	0.018	51.10	1.97	0.227	13.99	2.00	0.289	6.28
SO23	1.68	0.017	147.28	1.82	0.174	16.94	1.79	0.271	8.42
SO65	1.91	0.027	40.46	1.96	0.160	10.04	2.01	0.302	3.66
CE2	1.88	0.008	56.81	1.96	0.122	8.68	1.93	0.229	5.66
CO38	-*	-	-	1.99	0.086	17.82	2.05	0.182	9.62
KN48	1.93	0.055	64.82	1.99	0.143	10.30	1.95	0.191	5.62
ZK39	1.61	0.013	42.44	1.99	0.144	17.64	2.00	0.149	10.16
ZK42	1.65	0.039	34.72	1.95	0.132	12.64	1.93	0.117	6.57
KN55	1.96	0.037	43.63	2.06	0.227	13.56	1.96	0.347	3.18
Mean value	1.79	0.032	54.18	1.91	0.169	13.34	1.90	0.246	6.76
SD	0.16	0.016	25.04	0.11	0.047	3.19	0.12	0.063	1.95
CV (%)	8.71	50.56	46.22	6.02	27.87	23.91	6.20	25.76	28.89

*: no growth was observed

At 30°C the best performances were observed for *Lat. curvatus* KN55 strain, that achieved a good A value (1.96 OD_{600}) with the highest μ_{max} (0.347 OD_{600}/h) and the shorter lag phase time (3.18 h), as well as at 20°C, followed by *Lat. sakei* strains SO65 and SN58. The highest maximum OD_{600} measured was reached by CO38 (2.05 OD_{600}), while SN34 was characterised by the lowest A (1.63 OD_{600}) and a long λ of 7.78 h.

Regarding samples incubated at 10°C, the data showed a widespread variability (Figure 3.14). Among them, 2M9 and SE30 were characterised by the best growth performances. The first strain growth was described by the highest μ_{max} (0.054 OD_{600}/h) and reached a good maximum OD_{600} level (1.92) with the shorter lag phase (33.81 h). In the case of *Lat. sakei* SE30, its growth was characterised by the higher OD_{600} value (2.04), achieved after 49.38 h and with a growth rate of 0.041 OD_{600}/h. On the contrary, the worst performance was evidenced for CE2, SO23 and SB2 strains. All these strains were characterised by a very low μ_{max} (0.008 OD_{600}/h, 0.017 OD_{600}/h and 0.013 OD_{600}/h, respectively). SO23 was characterised also by the longer lag phase duration (147.28

h). In addition, it is important to underline that at 10°C one strain, *Lat. sakei* CO38, was unable to grow after 200 h of incubation.

Finally, also these data were affected by a huge variability, described through the range of value for each estimated parameters: *A* ranged between 2.04 OD_{600} and 1.50 OD_{600} with CV of 8.71%, λ varied from 33.81 h to 147.28 h (CV 46.22%), while μ_{max} from 0.054 OD_{600}/h to 0.008 OD_{600}/h (CV 50.56%).

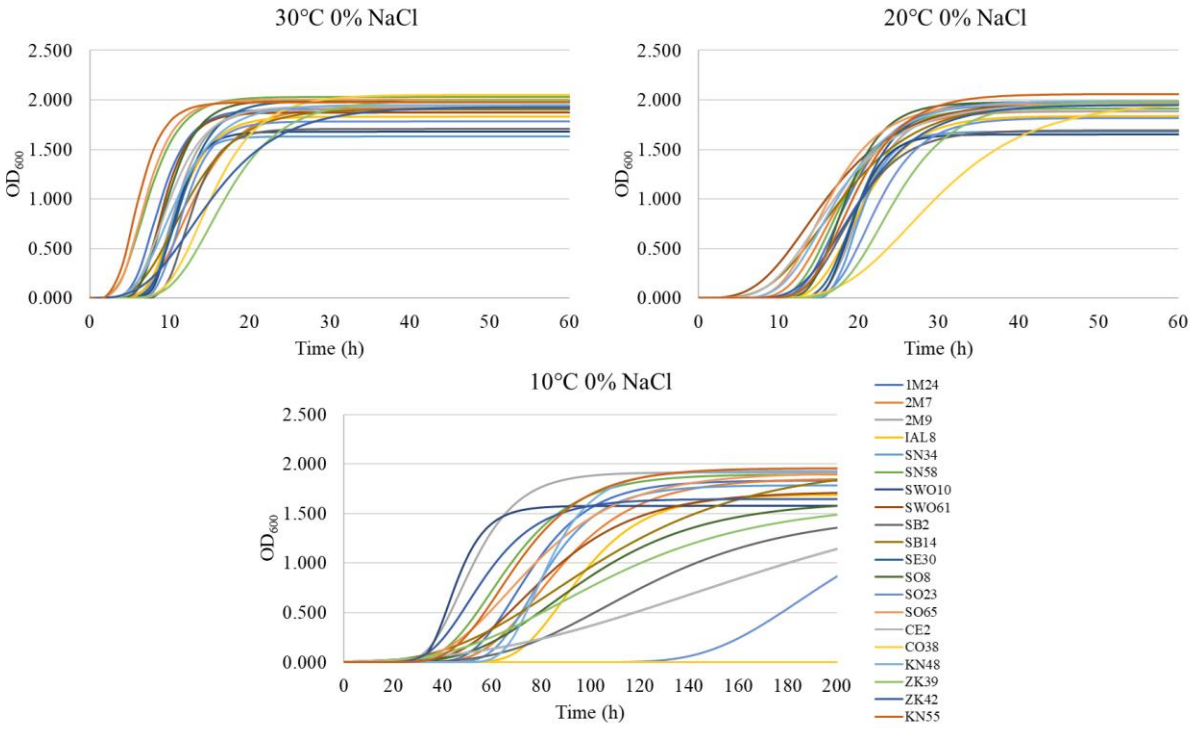


Figure 3.14: Growth curves at different incubation temperatures (10°C, 20°C and 30°C) obtained by using the estimated parameters of Gompertz equation.

To better underline the variability in strains growth performances, Box and Whisker plots were reported about data collected during analyses at different salt concentrations and at different incubation temperatures (Figure 3.15 and 3.16, respectively).

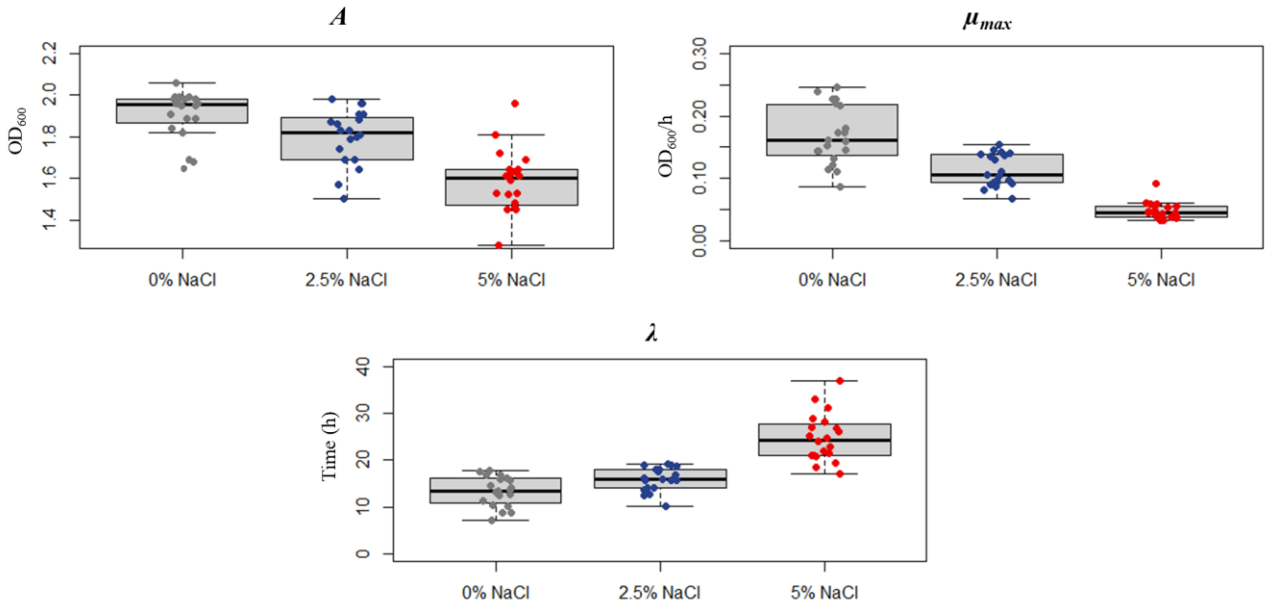


Figure 3.15: Box and Whisker plots that represent the distribution of parameters estimated by Gompertz equation about growth kinetics of strains at different salt concentrations (0%, 2.5% and 5%).

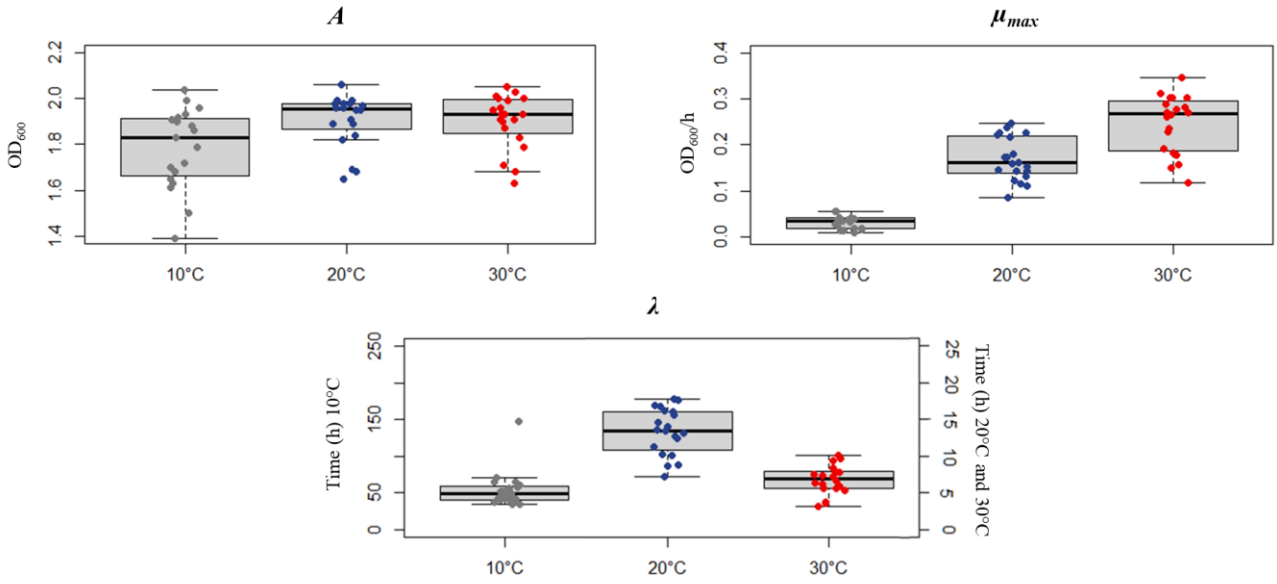


Figure 3.16: Box and Whisker plots that represent the distribution of parameters estimated by Gompertz equation about growth kinetics of strains at different incubation temperatures (10°C, 20°C and 30°C).

Moreover, for each sample incubated in each condition the pH was also monitored. Data collected were again modelled by using Gompertz equation and the estimated parameters (k, *A*, μ_{max} and λ) are reported in Table 3.25 and 3.26. In this case, the coefficient k was added to the equation in order to set the initial pH value, that was about 6.

As expected, results showed a specular behaviour with respect to the corresponding growth curve. In fact, the drop of pH depends on the growth performance of strains.

Table 3.25: Gompertz parameters (k, *A*, μ_{max} and λ) about *Lat. sakei* and *Lat. curvatus* strains acidification performances at 20°C in presence of different salt concentrations. Mean values, standard deviation (SD) and variability coefficient (CV) are also reported.

	pH 0%				pH 2.5%				pH 5%			
Strains	**k**	***A***	μ_{max}	λ	**k**	**A**	μ_{max}	λ	**k**	***A***	μ_{max}	λ
1M24	5.87	-1.84	-0.094	12.17	5.87	-1.55	-0.078	14.14	5.87	-1.38	-0.044	20.54
2M7	5.96	-1.90	-0.049	4.70	5.79	-1.69	-0.047	12.81	5.62	-1.52	-0.031	25.88
2M9	5.97	-1.58	-0.113	15.40	5.93	-1.40	-0.099	18.13	5.83	-1.23	-0.062	33.00
IAL8	5.95	-1.64	-0.121	16.18	5.90	-1.61	-0.103	17.21	5.87	-1.64	-0.048	23.37
SN34	6.01	-2.04	-0.077	14.05	5.98	-1.68	-0.081	17.96	5.82	-1.06	-0.067	35.73
SN58	5.90	-1.72	-0.090	12.87	5.80	-1.74	-0.048	12.48	5.80	-1.51	-0.029	19.11
SWO10	5.92	-1.44	-0.116	17.02	5.91	-1.37	-0.090	19.08	5.89	-1.38	-0.040	27.24
SWO61	6.06	-2.07	-0.056	1.86	5.81	-1.75	-0.046	7.52	5.62	-1.46	-0.028	16.72
SB2	5.90	-1.71	-0.075	13.40	5.84	-1.78	-0.059	16.95	5.82	-1.80	-0.033	28.47
SB14	5.92	-1.93	-0.056	5.73	5.79	-1.68	-0.050	12.96	5.77	-1.35	-0.031	22.12
SE30	6.09	-1.95	-0.082	12.87	6.03	-1.96	-0.058	13.55	5.99	-1.76	-0.037	20.66
SO8	5.86	-1.69	-0.133	14.92	5.85	-1.70	-0.080	14.75	5.84	-1.62	-0.030	21.14
SO23	6.00	-1.92	-0.094	15.60	5.94	-1.83	-0.066	16.35	5.94	-1.71	-0.043	22.38
SO65	6.12	-2.12	-0.082	6.71	5.80	-1.80	-0.061	11.16	5.75	-1.62	-0.039	20.13
CE2	5.88	-1.87	-0.065	8.62	5.78	-1.70	-0.063	13.58	5.76	-1.66	-0.034	20.33
CO38	6.05	-1.96	-0.083	17.41	5.98	-1.97	-0.063	15.33	5.92	-1.89	-0.042	18.06
KN48	5.99	-1.79	-0.120	13.24	5.97	-1.84	-0.077	12.62	5.91	-1.66	-0.038	16.10
ZK39	6.03	-1.81	-0.086	16.82	5.96	-1.88	-0.061	15.23	5.93	-1.82	-0.038	18.03
ZK42	6.01	-1.84	-0.098	12.58	5.97	-1.87	-0.070	15.02	5.92	-1.71	-0.038	18.80
KN55	6.13	-2.14	-0.105	10.61	5.84	-1.83	-0.083	14.36	5.71	-1.63	-0.043	25.27
Mean value	5.98	-1.85	-0.090	12.14	5.89	-1.73	-0.069	14.56	5.83	-1.57	-0.040	22.65
SD	0.08	0.18	0.023	4.44	0.08	0.16	0.017	2.70	0.10	0.21	0.010	5.24
CV (%)	1.37	9.86	26.12	36.56	1.35	9.24	24.26	18.58	1.74	13.37	25.47	23.13

Table 3.26: Gompertz parameters (k, *A*, μ_{max} and λ) about *Lat. sakei* and *Lat. curvatus* strains acidification performances at different incubation temperatures. Mean values, standard deviation (SD) and variability coefficient (CV) are also reported.

	pH 10°C				pH 20°C				pH 30°C			
Strains	**k**	***A***	μ_{max}	λ	**k**	***A***	μ_{max}	λ	**k**	***A***	μ_{max}	λ
1M24	5.88	-1.53	-0.012	24.10	5.87	-1.84	-0.094	12.17	5.94	-2.09	-0.131	3.93
2M7	5.92	-1.36	-0.025	62.07	5.96	-1.90	-0.049	4.70	5.74	-1.83	-0.090	7.83
2M9	5.90	-0.93	-0.050	38.98	5.97	-1.58	-0.113	15.40	5.87	-1.78	-0.170	7.79
IAL8	5.90	-1.39	-0.022	71.56	5.95	-1.64	-0.121	16.18	5.87	-1.75	-0.177	8.36
SN34	5.93	-1.31	-0.021	64.11	6.01	-2.04	-0.077	14.05	5.90	-2.03	-0.146	7.85
SN58	6.00	-1.70	-0.022	40.64	5.90	-1.72	-0.090	12.87	6.00	-1.86	-0.265	5.56
SWO10	5.93	-0.75	-0.071	41.04	5.92	-1.44	-0.116	17.02	5.92	-1.80	-0.168	8.37
SWO61	5.89	-1.35	-0.020	52.18	6.06	-2.07	-0.056	1.86	5.88	-1.95	-0.079	3.09
SB2	5.98	-1.50	-0.013	62.61	5.90	-1.71	-0.075	13.40	5.92	-1.70	-0.249	10.62
SB14	6.04	-1.52	-0.015	35.27	5.92	-1.93	-0.056	5.73	5.98	-2.10	-0.109	7.05
SE30	6.04	-1.47	-0.023	47.85	6.09	-1.95	-0.082	12.87	6.00	-1.98	-0.117	6.78
SO8	5.95	-1.73	-0.011	35.22	5.86	-1.69	-0.133	14.92	5.90	-1.73	-0.328	7.64
SO23	5.93	-1.39	-0.014	159.61	6.00	-1.92	-0.094	15.60	6.18	-1.88	-0.132	5.55
SO65	6.09	-1.77	-0.026	43.09	6.12	-2.12	-0.082	6.71	6.12	-2.03	-0.247	4.40
CE2	5.96	-1.47	-0.011	65.38	5.88	-1.87	-0.065	8.62	5.98	-2.06	-0.135	5.35
CO38	5.98	-*	-	-	6.05	-1.96	-0.083	17.41	5.99	-2.10	-0.153	9.45
KN48	6.07	-1.73	-0.025	17.80	5.99	-1.79	-0.120	13.24	6.04	-1.98	-0.127	2.99
ZK39	6.04	-1.66	-0.011	24.65	6.03	-1.81	-0.086	16.82	6.02	-1.89	-0.099	5.61
ZK42	6.02	-1.58	-0.024	24.43	6.01	-1.84	-0.098	12.58	5.99	-1.81	-0.152	10.49
KN55	6.14	-1.77	-0.024	41.57	6.13	-2.14	-0.105	10.61	6.10	-1.99	-0.271	3.81
Mean value	5.98	-1.47	-0.023	50.11	5.98	-1.85	-0.090	12.14	5.97	-1.92	-0.167	6.63
SD	0.08	0.27	0.015	30.80	0.08	0.18	0.023	4.44	0.10	0.13	0.069	2.31
CV (%)	1.26	18.28	63.29	61.46	1.37	9.86	26.12	36.56	1.68	6.81	41.09	34.84

*: no growth was observed

3.3.3.5 Aroma profile of selected LAB strains inoculate in a real food system

The selected LAB strains were also inoculated singularly in a meat batter, to mimic a real food system, and incubated at 20°C for 48 h. The samples were analysed to detect their content in volatile organic compounds (VOCs). Results are reported in Table 3.27 and Figure 3.17. Aroma profile was monitored using SPME-GC-MS technique and the VOCs detected were expressed as ratio between peak area of each molecule and peak area of the internal standard (4-methyl-2-pentanol), which was added in constant amount (3.3 mg/kg). The detected compounds are grouped in chemical classes (*i.e.* aldehydes, ketones, alcohols, acids and esters).

Despite the use of an internal standard, SPME technique was considered in this work as a qualitative tool to study the effect of inoculated strain metabolisms on the changes in meat volatile profile. In this perspective, the aim was to evidence qualitative modifications of VOCs presence and not to quantify them. In fact, the quantitative results obtained by this analytical technique depends

on several factors, among which the type of fibre and the time and temperature of absorption are the most important, which can strongly influence the amounts of each single molecules recovered.

The data showed that the detection of these compounds varied in relation to the different strains. For the 2 *Lat. sakei* CO38 and ZK42, the VOCs production was scarce and they were not able to accumulate important aroma molecules. The other strain aroma profiles presented significant amounts of aldehydes, consisting mainly of hexanal and, secondarily, of heptanal and 2-nonenal, which can result from the autoxidation processes of the lipid component of meat. Even if LAB should not have a direct role on this process, it seemed that they could exert an indirect effect, determining a change in its kinetics.

As regards ketones, they significantly varied according to the strain, being mainly represented by 2-pentanone, 2-heptanone, diacetyl, acetoin, 2,3-octanedione and acetone. Some of these molecules are certainly the result of microbial activity, particularly diacetyl and acetoin. The genesis of the other ketones, and in particular methyl-ketones, can be attributed either to microbial activities, including those conducted by LAB, or to autoxidation processes.

Among alcohols, whose presence was extremely variable in relation to the LAB strains, ethanol was a minor component, while the most conspicuous contributions were determined by 1-pentanol, 1-hexanol, 1-heptanol and 1-octen-3-ol. These latter molecules mostly derive from the reduction of the corresponding aldehydes resulting from the autoxidation processes.

Among acids, the greatest contribution was certainly given by the presence of acetic acid and, secondly, of hexanoic and butanoic acid.

Finally, among esters, the only one detected was n-caproic acid vinyl ester, which is reported in pork meat.

Based on the obtained results, it can be stated that the inoculated LAB, at least in the conditions adopted, showed a fairly low production of microbial VOCs, underlining that the contribution of lactobacilli to the formation of the fermented meat aroma profile is rather small, limiting to the production of some important molecules such as diacetyl, acetoin, acetic acid and ethanol.

On the other hand, in this screening procedure the results were acquired after 48 h of incubation in meat model and cannot reflect the overall result in an industrial production, where several factors can play important roles. In any case, the different strains, belonging mostly to *Lat. sakei* species, seem to have a different role in the overall trend of the oxidation process and in the generation of the general aroma profile in fermented meat.

In conclusion, among the tested strains, a great variability was observed in the production of some compounds, *i.e.* acetoin, diacetyl and acetic acid, which can be considered key aroma

molecules for sausage aroma profile. In addition, a lack of compounds derived from the metabolism of amino acids, except for limited amounts of 3-methyl butanoic acid, was highlighted. This can be due to the limited incubation time used in this trial.

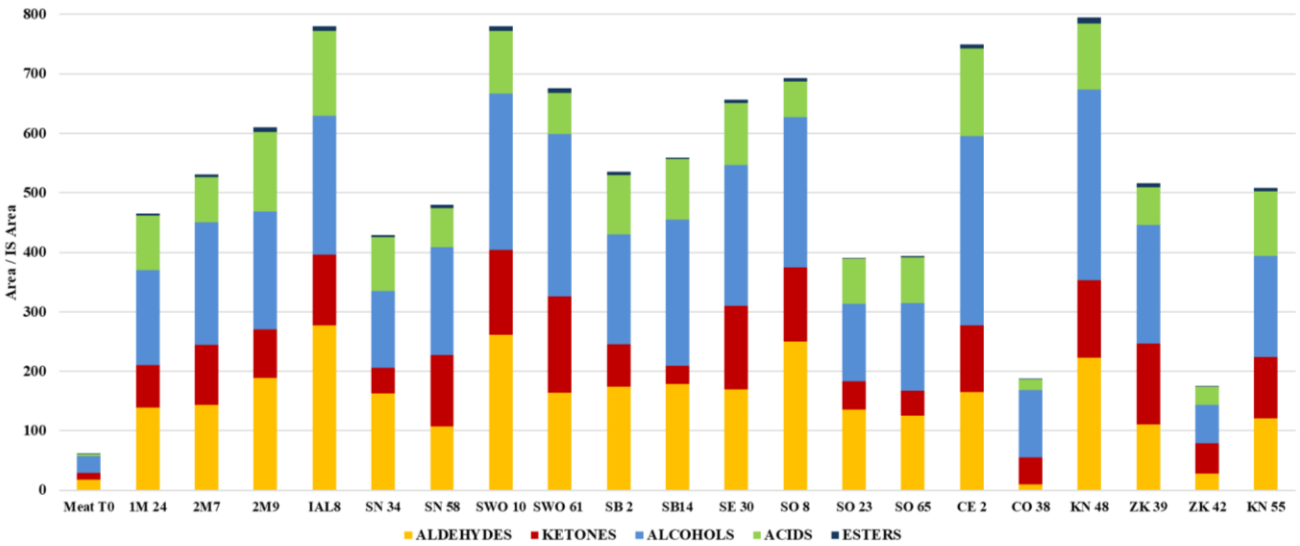


Figure 3.17: Presence of the different classes of volatile organic compounds (VOCs) in the samples. The values are expressed as the ratio between the peak area of the compound considered and the area of the internal standard.

Table 3.27: Volatile organic compounds (VOCs) detected by SPME-GC-MS in the samples, expressed as ratio between peak area of each molecule and peak area of the internal standard (4-methyl-2-pentanol). The standard deviation was always below 5%.

Compounds	**Meat T0**	**1M24**	**2M7**	**2M9**	**IAL8**	**SN34**	**SN58**	**SWO10**	**SWO61**	**SB2**	**SB14**	**SE30**	**SO8**	**SO23**	**SO65**	**CE2**	**CO38**	**KN48**	**ZK39**	**ZK42**	**KN55**
Pentanal	-*	-	1.28	7.95	-	4.20	-	-	-	-	7.11	-	-	4.74	2.25	7.37	-	-	-	0.47	-
Hexanal	15.32	112.01	104.17	125.00	211.05	128.62	77.87	198.56	97.47	134.62	140.15	130.98	200.17	102.41	94.95	101.21	2.51	168.27	80.46	15.33	80.95
Heptanal	0.44	10.25	11.71	16.26	22.09	11.03	8.38	21.61	21.61	14.04	8.91	11.80	17.69	8.51	8.78	12.05	-	14.78	8.11	2.12	11.46
Octanal	0.38	5.17	5.67	11.53	11.19	4.62	5.66	10.31	11.32	7.29	4.90	8.19	6.97	3.94	3.48	12.45	1.78	9.42	3.70	1.44	7.49
Nonanal	1.23	8.18	13.29	18.86	22.90	9.14	11.24	21.74	22.61	12.34	10.90	12.84	16.28	10.15	10.48	21.48	4.42	20.80	11.53	5.88	14.89
Decanal	0.29	-	1.63	1.93	2.27	0.64	0.49	1.60	1.92	0.00	1.29	0.00	1.51	1.56	1.63	2.46	-	0.84	1.52	1.01	-
2-nonenal	0.22	2.13	2.71	4.50	4.03	2.15	2.36	4.60	5.52	3.83	2.09	3.01	3.94	1.48	1.87	4.29	0.81	5.95	3.46	0.97	3.40
Benzaldehyde	0.39	1.80	2.62	3.17	3.71	2.15	1.70	3.36	3.27	2.38	3.39	2.33	2.90	3.01	1.81	3.52	-	3.11	2.08	0.78	2.23
Aldehydes	**18.28**	**139.53**	**143.08**	**189.19**	**277.23**	**162.55**	**107.70**	**261.78**	**163.71**	**174.49**	**178.73**	**169.16**	**249.46**	**135.79**	**125.25**	**164.84**	**9.53**	**223.18**	**110.86**	**28.01**	**120.43**
Acetone	3.29	6.04	10.88	8.09	6.62	5.99	9.47	11.41	11.43	6.82	4.81	10.24	10.82	4.24	2.16	9.51	3.72	9.38	8.52	3.65	7.81
2-butanone	0.59	0.94	1.43	1.24	1.39	0.79	1.30	1.51	1.74	1.23	1.09	1.60	1.60	1.54	0.94	26.18	6.78	1.40	6.69	2.53	1.30
2,3-butanedione	0.20	3.56	2.89	2.43	8.39	3.82	2.43	5.16	3.64	1.49	2.14	2.22	1.98	7.84	2.20	2.16	1.04	7.75	10.34	4.51	2.96
2-pentanone	1.18	24.12	36.88	21.33	46.20	6.08	61.43	61.62	90.00	31.09	1.70	75.30	68.51	1.41	11.19	30.04	3.84	46.09	50.22	1.57	37.50
Methyl isobutyl ketone	1.50	1.22	0.63	2.72	0.85	0.99	0.77	0.89	0.82	3.81	3.45	1.05	0.71	1.19	1.07	3.50	1.27	0.64	0.94	1.49	0.73
2,3-pentanedione	-	0.32	0.81	0.59	0.77	0.76	0.53	0.46	0.81	1.61	0.33	0.47	0.66	0.32	0.58	0.84	-	0.70	0.61	0.16	0.54
3-penten-2-one, 4-methyl	0.53	2.52	1.33	1.85	2.36	1.70	-	-	3.03	0.89	-	2.45	1.18	1.44	1.42	4.43	1.99	0.91	1.69	0.58	3.51
2-heptanone	0.68	4.60	10.98	8.22	10.99	3.78	11.78	12.87	17.96	7.92	4.66	16.48	11.28	4.13	3.25	15.13	4.97	12.07	9.78	2.10	11.97
2-octanone	0.62	1.62	3.16	2.82	3.57	1.30	2.91	5.04	5.35	3.01	1.33	4.21	4.06	1.46	1.25	3.39	1.71	3.81	2.86	1.15	4.39
2-butanone,3-hydroxy	0.54	19.55	12.72	19.34	27.38	11.10	15.07	26.92	13.94	2.28	3.65	15.30	8.68	11.52	10.57	7.87	15.98	29.94	32.25	29.85	16.61
2,3-octanedione	0.88	2.70	14.41	6.52	5.27	3.93	12.70	9.81	8.47	6.17	3.81	7.35	14.97	8.58	3.73	2.71	0.84	10.47	6.93	1.48	12.44
2-nonanone	0.07	0.73	0.78	0.98	1.44	0.52	0.74	1.43	1.40	1.19	0.79	1.22	1.07	0.39	0.61	1.19	0.37	1.86	1.22	0.51	1.05
3-decen-5-one, 2-methyl-	0.58	2.95	3.95	4.72	4.09	2.58	-	5.66	3.13	3.00	2.97	3.28	-	3.24	3.45	4.86	3.06	5.04	3.45	1.80	2.98
Ketones	**10.65**	**70.88**	**100.86**	**80.84**	**119.31**	**43.35**	**119.15**	**142.77**	**161.72**	**70.50**	**30.73**	**141.15**	**125.53**	**47.30**	**42.43**	**111.81**	**45.57**	**130.05**	**135.51**	**51.38**	**103.80**
Ethyl alcohol	1.05	1.98	3.02	2.38	1.85	1.80	1.87	8.98	4.35	3.24	5.57	1.68	4.81	1.38	2.17	2.77	7.11	9.45	7.33	3.22	3.83
1-propanol	-	0.94	-	0.89	0.86	-	-	-	-	-	-	-	-	-	-	2.48	0.28	-	-	-	-
1-butanol	0.50	1.27	1.71	1.22	1.86	0.97	3.32	1.59	2.15	1.16	0.72	2.55	2.21	0.68	1.17	2.48	-	3.14	1.41	0.28	3.07
1-penten-3-ol	0.47	2.54	2.69	2.88	3.76	2.96	2.24	4.18	2.98	3.32	1.97	3.09	3.32	1.58	2.22	3.84	2.31	3.14	3.64	0.74	2.09

1-butanol,3-methyl	0.32	1.01	2.71	-	-	0.39	-	2.41	-	-	-	1.68	2.10	0.21	0.37	-	0.38	1.60	1.49	0.43	0.60
1-pentanol	13.51	33.76	57.13	34.97	45.00	30.08	55.55	57.86	63.88	34.75	24.25	55.52	60.25	27.43	30.66	53.32	24.99	52.85	48.50	14.96	46.15
1-hexanol	3.42	68.70	63.81	69.78	83.68	44.39	51.89	86.21	92.96	61.88	106.79	89.30	96.26	48.82	67.44	148.13	48.45	142.03	65.88	22.41	50.66
1-octen-3-ol	5.46	28.79	42.01	52.54	54.62	30.53	36.76	58.37	55.11	42.03	46.51	48.35	45.30	34.42	25.24	62.73	14.69	50.12	34.01	10.75	37.52
1-heptanol	1.09	7.12	8.85	9.01	13.35	7.51	11.25	15.94	20.85	21.41	41.32	11.90	12.45	3.30	6.18	14.46	6.69	23.74	15.01	4.60	10.31
1-octanol	0.74	6.84	8.39	11.05	13.97	6.43	7.32	14.53	12.19	8.96	8.60	10.54	9.56	5.68	5.12	14.30	4.12	16.19	9.83	3.95	8.40
2-octen-1-ol	0.98	3.23	6.27	6.87	6.36	0.67	5.36	7.37	7.64	4.34	4.64	2.97	7.04	2.27	1.62	4.49	1.31	7.37	4.96	0.69	0.76
1-nonanol	-	0.83	0.97	1.61	1.47	0.90	-	1.70	1.63	0.88	1.22	1.39	-	0.82	1.22	2.64	0.92	5.81	3.48	1.30	0.88
4-nonanol	0.53	2.76	9.10	5.44	5.93	2.20	6.29	2.87	9.60	3.34	3.69	7.57	9.03	3.56	2.94	7.32	1.70	5.18	4.27	1.02	5.38
Alcohols	**28.06**	**159.75**	**206.66**	**198.62**	**232.70**	**128.84**	**181.85**	**262.02**	**273.34**	**185.32**	**245.27**	**236.55**	**252.34**	**130.14**	**146.35**	**318.97**	**112.95**	**320.62**	**199.82**	**64.36**	**169.63**
Acetic acid	1.18	54.70	26.82	79.27	76.88	54.97	24.88	46.15	18.95	47.03	64.72	54.15	18.69	46.39	46.43	89.70	8.81	32.60	18.55	12.87	48.18
Propanoic acid	0.25	1.29	1.24	2.08	1.85	1.10	1.19	1.47	1.86	1.47	1.15	1.13	1.12	0.88	0.90	1.76	0.78	2.39	1.47	0.64	1.66
Butanoic acid	0.36	3.15	2.58	4.74	4.94	3.15	-	3.10	2.63	4.44	4.77	2.97	0.33	2.62	2.74	4.37	0.61	4.92	2.88	1.97	3.84
Butanoic acid,3-methyl	-	2.23	2.46	3.59	2.70	1.24	1.77	3.50	2.30	1.67	1.20	2.54	1.74	0.58	1.09	2.24	0.88	3.40	1.79	1.13	2.31
Pentanoic acid	0.37	2.71	3.37	4.11	4.74	2.88	2.25	4.46	3.89	3.84	2.89	3.55	2.46	2.27	2.30	4.31	1.86	5.06	3.38	1.55	3.50
Hexanoic acid	1.51	21.41	34.24	33.12	46.58	23.60	30.82	41.03	32.87	33.99	21.45	35.60	28.45	18.48	17.91	37.17	4.15	51.07	27.07	6.49	40.95
Heptanoic acid	-	1.20	1.20	2.06	1.94	1.15	1.14	1.19	1.53	1.41	0.93	1.28	1.05	0.99	0.86	1.57	-	1.97	1.43	0.91	1.38
Octanoic acid	-	1.74	1.41	2.05	1.45	0.48	0.98	1.94	1.99	1.21	1.53	1.40	1.65	1.38	1.30	2.03	1.25	2.08	1.87	1.45	1.64
Nonanoic acid	0.42	1.67	2.45	2.75	2.32	2.34	2.77	2.87	3.19	1.99	2.18	1.67	2.43	2.57	2.27	2.88	-	4.63	2.66	1.88	2.72
n-decanoic acid	-	1.12	-	-	-	-	-	-	-	2.91	0.92	-	1.57	-	1.79	0.96	-	2.77	2.11	1.29	2.38
Acids	**4.10**	**91.22**	**75.76**	**133.78**	**143.41**	**90.91**	**65.79**	**105.70**	**69.20**	**99.96**	**101.74**	**104.28**	**59.48**	**76.15**	**77.59**	**146.99**	**18.35**	**110.88**	**63.20**	**30.17**	**108.57**
n-caproic acid vinyl ester	0.20	3.39	5.03	8.02	7.53	3.21	4.89	7.86	7.94	4.76	2.55	5.15	5.92	1.57	2.55	7.15	1.26	10.27	6.90	1.43	5.57
Esters	**0.20**	**3.39**	**5.03**	**8.02**	**7.53**	**3.21**	**4.89**	**7.86**	**7.94**	**4.76**	**2.55**	**5.15**	**5.92**	**1.57**	**2.55**	**7.15**	**1.26**	**10.27**	**6.90**	**1.43**	**5.57**

*: not detected under the adopted conditions

3.3.4 Conclusions

In this part of the thesis, 46 LAB strains tested as not aminobiogenic and not antibiotic-resistant, belonged mainly to *Lat. sakei* and only one to *Lat. curvatus* species, were studied to select potential autochthonous starter or bio-protective cultures.

The strains were firstly evaluated for their antimicrobial activity against *E. coli* ATCC 25922 and *List. innocua* UC 8409. Collected data showed that all tested strains were able to inhibit the growth of both target microorganisms, even if at different levels depending on the specific strain. In general, *E. coli* represented the most sensitive microorganism to LAB inhibition, while *List. innocua* was hindered less severely. Moreover, the antimicrobial activity of LAB strains against the same target microorganisms was also evaluated in a meat model. The data confirmed a greater sensitivity of *E. coli* with respect to *List. innocua*, but the most effective strains were able to inhibit both the target pathogens tested. Given these antimicrobial activities, bacteriocins encoding genes were searched. 24 *Lat. sakei* strains and the *Lat. curvatus* were characterised by the presence of at least one bacteriocin encoding gene, mainly sakacin X and sakacin P. These strains demonstrated the highest antimicrobial activity against target microorganisms *in vitro* and a good potential in meat model.

The most promising strains, characterised by the best antimicrobial performances, were selected for further technological analyses. In particular, the growth of 20 LAB strains (19 *Lat. sakei* and 1 *Lat. curvatus*) was monitored at different salt concentrations (0%, 2.5% and 5%) and at different incubation temperatures (10°C, 20°C and 30°C). As expected, a growth potential reduction was observed when the salt concentration increased or the temperature decreased, with lower growth rates and increasing in lag phase. A great variability in the strain behaviour has been evidenced, confirming the phenotypic diversity already reported for LAB species highly adapted to meat environment.

In addition, their aroma profile in a real food system was also determined, in order to evaluate their possible role on organoleptic characteristics. The results showed a significant variability in the composition of aroma compounds related to the strains. In particular, some of them were characterised by a low accumulation of these compounds and this could make them possible candidates to be used as a bio-protective cultures, due to their low organoleptic impact.

Based on these analyses, it was possible to identify some promising LAB strains characterised by suitable technological features and antimicrobial potential to be proposed as starter or bio-protective cultures in real food systems, such as fresh or fermented sausages, with the aim to improve food quality and microbiological safety, as well as giving peculiar characteristics to the final product.

Work under submission as:

Barbieri, F., Tabanelli, G.*, Montanari, C., Gardini, F., Belloso Daza, M.V., Milani, G., Cocconcelli, P.S., Bassi, D. Functional and technological characterisation of LAB strains isolated from Mediterranean spontaneously fermented sausages. Under preparation to be submitted to *LWT - Food Science and Technology*.

Chapter 4:

Use of new LAB cultures in meat products: evaluation of their bio-protective activities and technological performances

4.1 Application of autochthonous LAB as bio-protective cultures in fresh sausages

4.1.1 Introduction

Among fresh traditional meat products, Italian pork fresh sausages are obtained by mixing of lean pork meat and fat in a ratio and at a mincing level depending on the traditional recipes and the geographical area of production (Raimondi et al., 2018). Moreover, spices (*i.e.* black pepper, garlic, etc.), water, salt, sugars (generally glucose or sucrose), wine and few acidifying and/or antioxidant preservatives can be added (European Commission, 2004). Meat batters are stuffed in natural or synthetic casings with variable diameters, often packaged under modified atmosphere and stored under refrigeration conditions (0-4°C) (Raimondi et al., 2018). Since no thermal treatment can be applied during the production and given the product characteristics (*i.e.* high a_w and pH), fresh sausages are particularly susceptible to microbial proliferation and the shelf-life is rather short at 4°C (10-15 days). The bacteria responsible for spoilage of fresh sausage are mainly LAB, such as carnobacteria, lactobacilli and leuconostocs, and *Brochothrix thermosphacta*, a facultative anaerobe bacterium that is considered one of the main spoilage agents in raw meat stored aerobically or in modified atmosphere. However, even if fresh sausages require coking before consumption, they are considered potential vehicles of food-borne pathogens transmission, such as *Salmonella, Staph. aureus* and *List. monocytogenes*. In fact, for this reason, the European Union regulation 2073/2005 (European Commission, 2005) does not give any indication concerning the presence of the pathogen in this product, although it represents a concern for their safety.

List. monocytogenes is often described as associated with RTE, meat and dairy products, being ubiquitous in the environment and able to survive and grow at low temperatures, a wide range of pH, high salt concentrations, low a_w and to adhere to many surfaces and form biofilms (de Castilho et al., 2020). *List. monocytogenes* is resistant to a variety of adverse conditions and can persist in food-processing environments, causing recurrent contamination of final products. For these reasons, the control of *List. monocytogenes* in foods is a constant challenge for the food industry, especially in RTE meat products that are consumed without additional heat treatments. The reported worldwide listeriosis incidence ranged from 0.3 to 0.48 cases/100000 persons during 2008 and 2018, indicated 2549 confirmed cases of invasive listeriosis in humans in the European Union in 2018, with a fatality rate of 15.6% (EFSA, 2021). High concentrations of this pathogen have been reported in raw pork meat and in chicken meats (Li, et al., 2018). Between 2017 and 2018, in South Africa not-fermented sausages produced by an industry whose production plant was colonized by *List. monocytogenes* determined a large outbreak with more than 1000 hospitalisation

and at least 216 deaths (Tchatchouang, et al., 2020). Recently, a serious *Listeria* outbreak was reported in Italy, connected with the presence of this pathogen in sausages made with poultry meat produced by an Italian company and in its production plant.

Another notable cause of outbreaks associated with fresh meat product is represented by *Salmonella* spp. Contamination of fresh sausages by this pathogen were reported in Brazil products (Cabral et al., 2014) and more recently in raw Danish pork sausage (Helmuth et al., 2019), making these products a potential health hazard for consumers.

Besides to appropriate hygiene practices during production and high raw material microbiological quality, innovative approaches such as the use of bio-protective strains or bacteriocins have been investigated for the control of food-borne pathogens contamination of fresh sausages. In fact, it has been demonstrated that the use of bacteriocinogenic strains or bacteriocins can effectively contribute to meat products' safety, especially when integrated into hurdle concepts (Woraprayote et al., 2016). In the recent years, several research have been addressed to the characterisation and the selection of new bacteriocinogenic LAB strains to be proposed for meat sector applications (Bhattacharya et al., 2022; Bintsis, 2018).

In this part of the PhD thesis, two promising LAB strains, isolated from spontaneously Spanish fermented sausages in the frame of the EU PRIMA project BioProMedFood, were studied. These strains had been previously genetically identified and characterised for their *in vitro* antimicrobial activity against food-borne pathogens during the activities of Spanish project partner. Due to their bio-protective potential, they had been assessed for the production of proteinaceous antimicrobial compounds and the bacteriocins have been identified. In this work, these two promising strains, belonging to the species *Ltp. paraplantarum* and *P. acidilactici*, have been tested for their antimicrobial activities against *List. monocytogenes* and *S. enterica* serovar Enteritidis *in vitro* determining the growth dynamics of the pathogens when co-cultured with LAB. In addition, the effects of the two LAB strains, inoculated at different concentrations, were evaluated in challenge tests in fresh sausages against the same pathogens tested before, to study their antimicrobial activity in food model in laboratory scale trials.

4.1.2 Materials and methods

4.1.2.1 LAB isolated form Spanish spontaneously fermented sausages and their identification

Different presumptive LAB isolates have been obtained from spontaneously fermented sausages produced in Andalusia, Extremadura and Murcia region (Spain). These strains have been

characterised with the aim to select new promising strains to be used as starter or bio-protecting cultures in meat industry.

Preliminary identification of LAB biotypes was based on strains phenotypic characteristics, including cell morphology, Gram-staining, catalase activity and their ability to grow at 10 and 45ºC and in the presence of 6.5% (w/v) of salt. Afterwards, total genomic DNA was extracted from pure culture according to Martín-Platero et al. (2007) and amplified by PCR by using universal primers 616V (5'-AGAGTTTGATYMTGGCTCAG-3') and 699R (5'-RGGGTTGCGCTCGTT-3'). PCR products were checked by electrophoresis on a 1% agarose gel. The sequencing of the PCR fragments was carried out on an ABI 3730xl sequencer (Applied Biosystems, Madrid, Spain) using the same primers at a concentration of 5 nM. 16S rRNA gene sequence analysis was performed using EzTaxon (Kim et al., 2012b) and BLAST (Altschul et al., 1997) platforms. The complete genome was sequenced with the Illumina HiSeq4000 platform by STAB VIDA (Caparica, Portugal). To perform the comparative genomic analysis, Artemis (Carver et al., 2012) and BLAST (Altschul et al., 1990) were used.

4.1.2.2 Antimicrobial activity against food-borne pathogens

The antimicrobial activity of identified biotypes (151) was evaluated through an agar spot test against several food-borne pathogens and spoilage microorganisms: *List. monocytogenes* CECT 4032, *Staph. aureus* CECT 976, *Cl. perfringens* CECT 563 and *Ent. faecalis* S-47. Target microorganisms were grown in BHI medium (Oxoid), while LAB strains into MRS (Oxoid). Each culture was incubated overnight at 30°C. Pathogen and spoilage microorganisms were spread in BHI agar plates in order to obtain a bacterial lawn with a final concentration of 6 log cfu/ml. Once the plate was dried, 10 µl of each strain were spotted onto plates. The samples were observed after 24 h of incubation at 30°C and the absence/presence of inhibition zones was evaluated. The inhibitory activity was expressed based on the diameter halo around the spot: + (5-10 mm), ++ (10-15 mm), +++ (> 15 mm), or - (no halo). Two independent tests were carried out, and each sample was tested in duplicate.

4.1.2.3 Bacteriocins production and identification

Strains that showed a good antimicrobial activity were evaluated for the possible production of bacteriocins. Briefly, 1 l of 0.1 M BHI medium in phosphate buffer was separately inoculated at 2% with fresh cultures of strains of interest (in stationary phase) and incubated overnight at 30°C. Then, suspensions were centrifuged at 4°C for 20 min at 4000 rpm to collect supernatants to be tested by the well technique (Tagg and McGiven, 1971) (Figure 4.1).

Moreover, to evaluate the sensitivity of the inhibitory substances to proteolytic enzymes, the well-diffusion agar assay was repeated after cell-free supernatant treatments with proteinase K (1 mg/ml), papain (1 mg/ml) and trypsin (1 mg/ml) at 37°C for 2 h.

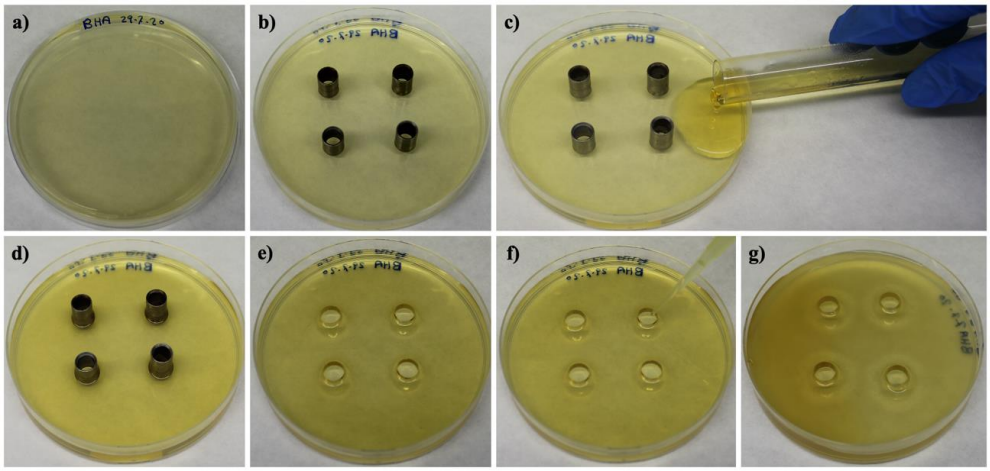


Figure 4.1: Well diffusion method, according to Tagg and McGiven (1971): a) BHA plate agar, b) stainless-steel cylinders, c) addition of BHI broth inoculated with target microorganism, d) solidification of the overlayer, e) removal of stainless-steel cylinders, f) LAB supernatant spotting, g) measurement of the halo diameter around the well.

Samples that showed an antimicrobial activity caused by the presence of a peptidic-nature bio-active compounds were purified through a cation exchange chromatography, with a two-step procedure based on the bacteriocin adsorption/desorption on Carboxymethyl Sephadex CM-25 (Sigma Aldrich), followed by a reverse-phase chromatography on a semi-preparative column (Abriouel et al., 2003). Then, samples were separated with a solution characterised by one volume of 0.5 M NaCl and two of 1.5 M NaCl. Further, the initial culture supernatant, the sample decanted and the same eluted with NaCl solutions were again tested using the well technique to determine their activity.

Samples that showed antibacterial activity after the cation exchange chromatography were re-purified by reversed-phase extraction on a C-18 solid support and tested against target microorganisms. Those that showed antimicrobial activity were lyophilised, re-suspended in 0.1% TFA MiliQ water and they were subjected to reverse-phase high performance liquid chromatography (RP-HPLC). The Agilent 1100 analytical chromatographic equipment a 5 µm C-18 column were used.

Finally, mass determination of antimicrobial compounds produced by strains as obtained by matrix-assisted laser desorption ionization-time of flight mass spectrophotometer MALDI-TOF MS AUTOFLEX® (Bruker Daltonics Inc., Billerica, USA) from the Centre for Scientific Instruments (CIC) of the University of Granada.

4.1.2.4 *In vitro* test to evaluate the growth of *List. monocytogenes* and *S. enterica* serovar Enteritidis in co-cultures with LAB strains

To assess LAB antimicrobial activity, the most promising strains (*Lactiplantibacillus paraplantarum* BPF2 and *Pediococcus acidilactici* ST6), were inoculated at a concentration of about 6 log cfu/ml in BHI medium (Oxoid) and co-cultured with *Listeria monocytogenes* Scott A and *Salmonella enterica* serovar Enteritidis 155, inoculated at 3 log cfu/ml. LAB and Pathogen strains were stored in MRS or BHI medium (Oxoid), respectively, with 20% (w/v) glycerol at -20°C and, before analyses, they were pre-cultivated twice. In particular, LAB strains were pre-cultivated in MRS broth at 30°C for 24 h, while pathogen strains in BHI medium at 37°C for 24 h.

The co-cultures were incubated at 20°C and strains' growth was monitored over time (up to 78 h) by plate counts onto LSO to detect *List. monocytogenes*, XLD to assess *Salmonella* growth and MRS for LAB strains enumeration.

Data obtained (plate counts) were modelled with the Gompertz equation (Zwietering et al., 1990):

$$y = k + A \cdot e^{-e^{\left[\left(\frac{\mu_{max} \cdot e}{A}\right)\cdot(\lambda - t)+1\right]}}$$

where y is the cellular concentration at time t, k is a constant representing the initial cellular load, *A* parameter represents the difference between maximum cellular load reached and the initial cell concentration, μ_{max} is the maximum log cfu/ml increase rate in exponential phase and the parameter λ is the lag phase duration (in hours).

Moreover, since in some samples an initial decrease of cell counts was observed due to antimicrobial LAB activity, a double-peaked Gompertz equation was used to fit also the first part of the curves (Tattershall et al., 2021).

$$y = k + \left(A_1 \cdot e^{-e^{\left[\left(\frac{\mu_{max1} \cdot e}{A_1}\right)\cdot(\lambda_1 - t)+1\right]}}\right) + \left(A_2 \cdot e^{-e^{\left[\left(\frac{\mu_{max2} \cdot e}{A_2}\right)\cdot(\lambda_2 - t)+1\right]}}\right)$$

In these case, y is the cellular load at time t, k is a constant representing the initial cellular load, *A*1 represents the difference between the initial inoculum and the cellular load reached in the first phase of decrease, *A*2 represent the difference between the minimum cellular load after the first phase of decrease and the maximum cellular load reached in the second growth phase, $\mu_{max}1$ and $\mu_{max}2$ are the maximum log cfu/ml decrease or increase rate in exponential phase, respectively, and the parameters $\lambda 1$ and $\lambda 2$ are the lag phase duration of the two phases. In both cases, k was maintained constant at 3 log cfu/ml as determined by plate counting immediately after the inoculum. Data modelling was performed using Statistica 8.0 software (Statsoft Inc.).

4.1.2.5 Challenge test in fresh sausages

The antimicrobial activity of the two selected strains, previously tested *in vitro*, was evaluated in a challenge test at a laboratory scale against the same food-borne pathogens, inoculated in a fresh sausage batter, produced by CLAI S.c.a, an Italian company (Figure 4.2).

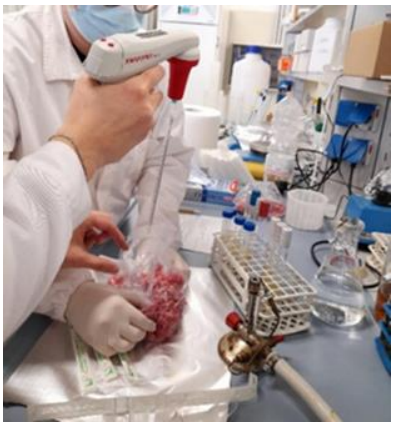


Figure 4.2: Meat batter inoculated during challenge test at a laboratory-scale.

The meat batter was divided into two batches to be inoculated with *List. monocytogenes* ScottA or *S. enterica* serovar Enteritidis 155, separately at a final concentration of about 3 log cfu/g. Then, samples were further aliquoted in different batches in which the selected LAB strains were inoculated at two different concentrations: 6 log cfu/g and 8 log cfu/g. Samples were stored at an abused temperature of 6°C and analysed immediately after the inoculation and after 1, 3, 6,10 and 13 days of storage to enumerate the inoculated microorganisms by sampling method, described in the Paragraph 3.1.2.3, onto selective agar media (LSO, XLD or MRS). These analyses were performed in triplicate.

4.1.3 Results and discussion

4.1.3.1 Screening and selection of LAB strains isolated from Spanish spontaneously fermented sausages

Starting from 1086 LAB isolated by DOMCA from Spanish naturally fermented sausages, 151 biotypes were detected and identified, according the 16S rRNA gene sequences. They were evaluated through an agar spot test for their antimicrobial properties against several microorganisms characterised by a high food safety concern (*List. monocytogenes* CECT 4032, *Staph. aureus* CECT 976, *Cl. perfringens* CECT 563 and *Ent. faecalis* S-47). The positive antimicrobial activity was evidenced as a clear inhibition zone in target microorganisms' layers caused by 62 out of 151 strains, belonged to *Lat. sakei*, *Lat. curvatus*, *P. acidilactici* and *Ltp. paraplantarum* species. In particular, results of the two most interesting strains are reported in Table 4.1. For this reason, they

were chosen for further trials. These strains showed a particularly enhanced activity against *List. monocytogenes*, followed by *Cl. perfringens*, *Staph. aureus* and *Ent. faecalis*.

Table 4.1: Antimicrobial activity of the two most interesting strains isolated from Spanish spontaneously fermented sausages. Data are expressed as: - no activity, + low activity (5-10 mm), ++ moderate activity (10-15 mm), +++ high activity (> 15 mm).

LAB strains	*List. monocytogenes* CECT 4032	*Staph. aureus* CECT 976	*Cl. perfringens* CECT 563	*Ent. faecalis* S-47
***Ltp. paraplantarum* BPF2**	+++	+	++	+
***P. acidilactici* ST6**	+++	+	++	+

The strong anti-listerial activity of *Ltp. paraplantarun* BPF2 and *P. acidilactici* ST6 was presumably attributed to an extracellular inhibitor substance, probably bacteriocins. To confirm this hypothesis, the nature of these antimicrobial compounds was studied. In particular, three proteinase enzymes were used on the cell free supernatants (CFS) (papain, trypsin and proteinase K) and the antimicrobial effect of the CFS was evaluated against *List. monocytogenes* CECT 4032, previously used (Table 4.2). The loss of inhibition indicated that the nature of the inhibitory substances produced by LAB strains was peptide-based, *i.e.* bacteriocins.

Table 4.2: Inhibition halo (mm) against *List. monocytogenes* CECT 4032 after enzyme treatments (1 mg/ml).

LAB strains	Control	Papain	Trypsin	Proteinase K
***Ltp. paraplantarum* BPF2**	17	-*	-	-
***P. acidilactici* ST6**	18	-	-	-

*: no halo was observed

Moreover, to purify these compounds, strains were grown in BHI broth and components of supernatants were separated by cation exchange chromatography. Then, fractions showing antimicrobial activity were further purified by reversed-phase extraction on a C-18 solid support and the components separated with reverse-phase high performance liquid chromatography (RP-HPLC). The mass identification of antimicrobial compounds was carried out by MALDI-TOF.

In *Ltp. paraplantarum* BPF2 supernatant the presence of plantaricin A and a pediocin was detected, while *P. acidilactici* ST6 showed pediocin production, according to the identification.

Pediocins are bacteriocins belonged to class II, characterised by a not-modified amino acid hydrophobic peptides, heat stable, with a size less than 10 kDA, which are particularly effective against *List. monocytogenes* (Khorshidian et al., 2021; Porto et al., 2017). These compounds are produced by pediococci, but also by *Ltp. paraplantarum* (Loessner et al., 2003). Plantaricins are a wide group of bacteriocins, classified in class I and II, produced by *Ltp. paraplantarum* and related species and active against *List. monocytogenes* and other pathogens, including Gram-negative species (Kareem and Razavi, 2020).

4.1.3.2 Effect of selected LAB strains against *List. monocytogenes* and *S. enterica* serovar Enteritidis growth kinetics

After the identification of their produced bacteriocins, *Ltp. paraplantarum* BPF2 and *P. acidilactici* ST6 were co-cultured in BHI medium with *List. monocytogenes* ScottA and *S. enterica* serovar Enteritidis 155 to evaluate their antimicrobial activity. LAB strains were inoculated at a final concentration of 6 log cfu/ml and target microorganisms at approx. 3 log cfu/ml. A control inoculated with the pathogen strain alone was also monitored. The different samples were incubated for 78 h at 20°C, to simulate the environmental conditions that often occur during the ripening processes of fermented foods (cheese and fermented sausages), in which these pathogens may be present.

The data obtained from plate count at different sampling times were modelled through Gompertz equation to estimate the growth parameters (Tattershall et al., 2021; Zwietering et al. 1990). The results of the different growth kinetics are reported in Table 4.3 and Table 4.4 for *List. monocytogenes* and *S. enterica* serovar Enteritidis, respectively.

Table 4.3: *List. monocytogenes* Scott A growth parameters in the presence of LAB strains estimated by modelling the data from plate counting (log cfu/ml) with the Gompertz equation. The maximum cell load is also reported (log cfu/ml).

Strains	k	$A1$	$\mu_{max}1$	$\lambda 1$	$A2$	$\mu_{max}2$	$\lambda 2$	Max cell load
Control	3.04	5.88	0.157	4.03				8.23
P. acidilactici ST6	3.30	-2.27	-0.261	0.17	3.05	0.129	34.10	4.08
Ltp. paraplantarum BPF2	2.85	-*	-	-	-	-	-	2.85

*: no growth

Table 4.4: *S. enterica* serovar Enteritidis 155 growth parameters in the presence of LAB strains estimated by modelling the data from plate counting (log cfu/ml) with the Gompertz equation. The maximum cell load is also reported (log cfu/ml).

Strains	k	A	μ_{max}	λ	Max cell load
Control	3.03	6.04	0.354	3.56	9.07
P. acidilactici ST6	2.87	5.84	0.328	4.31	8.81
Ltp. paraplantarum BPF2	2.87	5.82	0.396	2.82	8.69

With respect to *S. enterica* serovar Enteritidis 155, data obtained for the inhibition of *List. monocytogenes* ScottA were more promising and interesting. In fact, in this case, the tested LAB strains were strongly effective in reducing the growth kinetics of this pathogen (Figure 4.3).

In both samples characterised by the co-culture with *P. acidilactici* ST6 and *Ltp. paraplantarum* BPF2, the final cell load was lower if compared to the control (8.23 log cfu/g *vs.* 4.08 or 2.85 log cfu/g, respectively with BPF2 and ST6 strains). However, the behaviour of *Listeria* growth kinetics when inoculated in co-culture with the two LAB strains was different, as well as the maximum cell load. It is noteworthy that the strain *Ltp. paraplantarum* BPF2 completely inhibited

List. monocytogenes, that was not able to actively grow during incubation. For this bacteriostatic effect, it was not possible to model the data obtained (Table 4.3).

On the contrary, *P. acidilactici* ST6 notably affected the growth kinetics of *Listeria* through an immediate decrease in its concentration of about 2.3 log cycle with respect to the initial concentration (3.3 log cfu/g). Then, after a further lag phase of 34 h, *List. monocytogenes* was able to reach a maximum cell load of 4 log cfu/g.

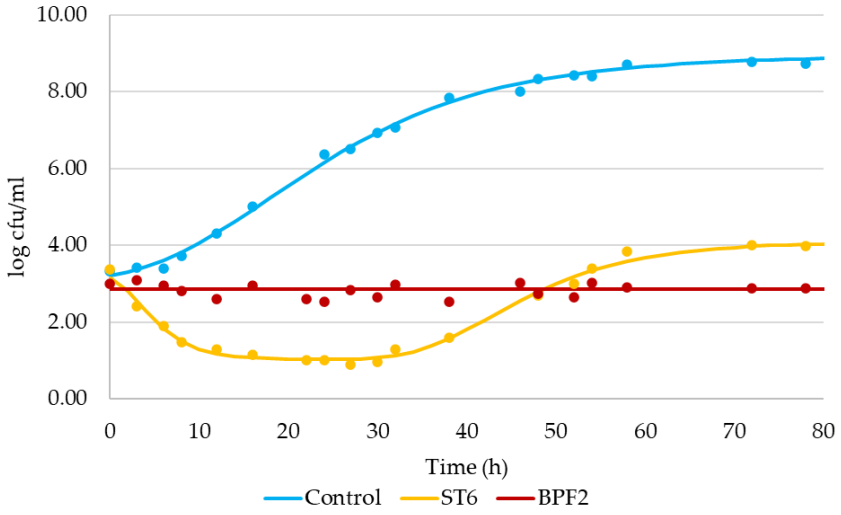


Figure 4.3: Growth kinetics of *List. monocytogenes* ScottA cultivated in the presence of different LAB strains. The points in the graph represent the experimental data collected from plate counting, while the curves are obtained through the estimated parameters by the model used.

On the other hand, no effect caused by the presence of selected LAB strains were observed against *Salmonella*. The behaviour of the target microorganism in these two samples (with the addition of ST6 and BPF2) was always comparable to the control. In fact, no significative differences were showed by the estimated parameters (Table 4.4) and the results are graphically reported in Figure 4.4.

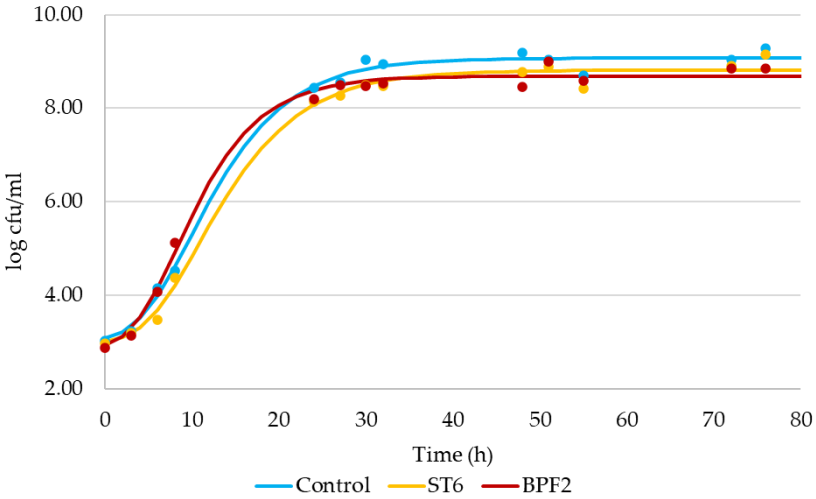


Figure 4.4: Growth kinetics of *S. enterica* serovar Enteritidis 155 cultivated in the presence of different LAB strains. The points in the graph represent the experimental data collected from plate counting, while the curves are obtained through the estimated parameters by the model used.

4.1.3.3 Challenge test against *List. monocytogenes* and *S. enterica* serovar Enteritidis in fresh sausages

Ltp. paraplantarum BPF2 and *P. acidilactici* ST6, previously studied in *in vitro* tests, showed a high activity in reducing the growth kinetics of *List. monocytogenes*, with respect to *S. enterica* serovar Enteritidis. However, both LAB strains were used in challenge tests in fresh sausages to confirm these results and to assess their antimicrobial activity in real food models against both pathogens (*List. monocytogenes* ScottA and *S. enterica* serovar Enteritidis 155).

LAB strains were inoculated as bio-protective cultures in meat batter, produced industrially according to the recipe, at a concentration of approx. 6 log cfu/g. Then, *List. monocytogenes* and *S. enterica* serovar Enteritidis were separately inoculated at an initial concentration of approx. 3 log cfu/g. The sausages were stored at 6°C, simulating a slight thermal abuse, and were analysed at time 0 and after 1, 3, 6, 10 and 13 days of storage to monitor the concentration of LAB, *Listeria* and *Salmonella*. Moreover, a control sample, obtained without the addition of any LAB strain was monitored, to assess the growth of target microorganisms.

In Figure 4.5, LAB microbial counts (as mean values of the two different challenge test) are reported. The initial concentration of lactobacilli in the control was 3.81 log cfu/g, due to meat and environmental contamination. After 3 days, this microbial group grew to approx. 5 log cfu/g, reaching after 6 days concentrations around 9 log cfu/g. In the samples containing *P. acidilactici* ST6 and *Ltp. paraplantarum* BPF2, LAB counts showed a slow increase in the first 3 days and reached concentrations comparable with the control after 6 days of storage. In effect, *P. acidilactici* growth could be affected by the low storage temperature and also *Ltp. paraplantarum* metabolism may suffer this refrigerate storage.

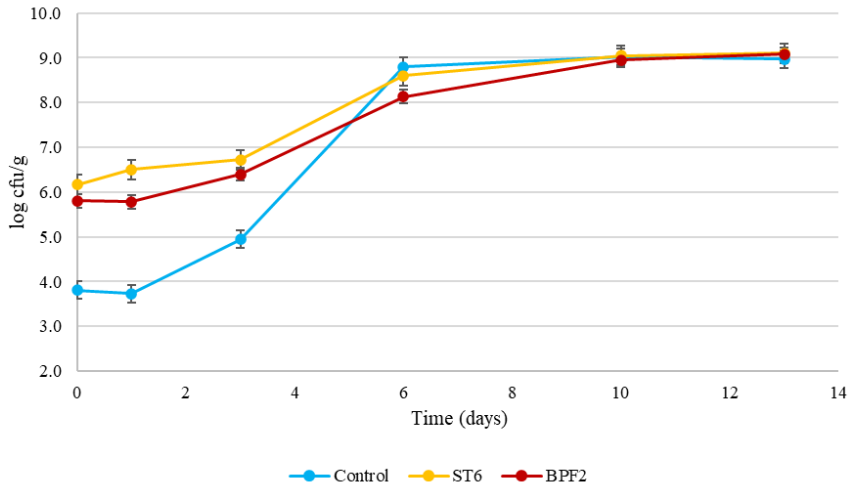


Figure 4.5: LAB microbial counts (log cfu/g) during the storage of fresh sausages at 6°C in the challenge test trial (initial inoculum of 6 log cfu/g). Standard deviation is also reported.

Concerning *List. monocytogenes* growth, each sample behaved rather similarly (Figure 4.6). In general, *Listeria* concentrations started to increase between 3 and 6 days and, at the end of storage (13 days), in all the samples pathogen counts reached values comprised between 6.34 log cfu/g and 6.47 log cfu/g, approx. 3 log cycles higher than the initial concentration.

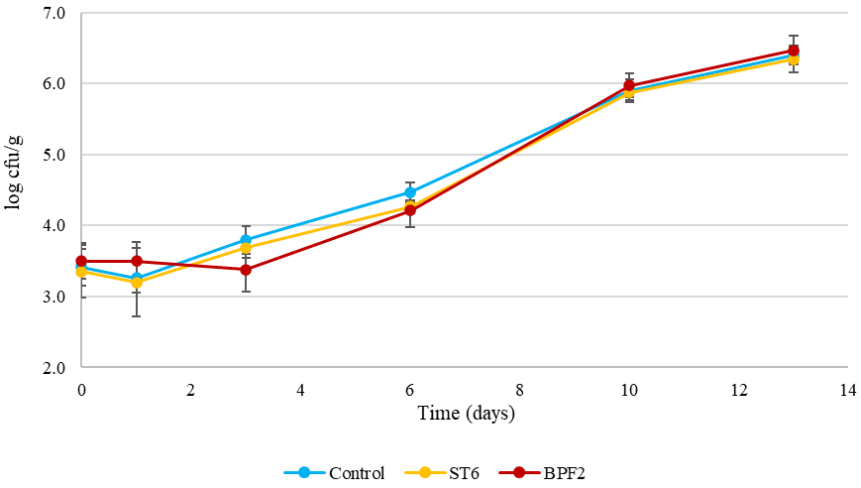


Figure 4.6: *Listeria monocytogenes* ScottA cell counts (log cfu/g) during storage of fresh sausages at 6°C in the challenge test trial, in which LAB strains were inoculated at a final concentration of 6 log cfu/g. Standard deviation is also reported.

The discrepancies observed in the application of these LAB strains in fresh sausages respect to *in vitro* results can be explained by the inhibition of the metabolism of *P. acidilactici* and *Ltp. paraplantarum* species exerted by low temperature. Indeed, the absence of growth does not necessarily mean that other biochemical ways are not active to guarantee, in any case, the survival

of the bacteria. However, the results obtained indicated that the pathway responsible for the antimicrobial peptide production does not work under these conditions.

Moreover, a challenge test against *S. enterica* serovar Enteritidis was performed in order to assess the behaviour of this pathogen in a real food system, even if the *in vitro* analyses demonstrated a scarce effect of LAB against this pathogen. In this case, the previously results were confirmed by the food model trails, in which *S. enterica* serovar Enteritidis cell load was not affect by the presence of LAB strains. Furthermore, neither an increase of this target microorganism was observed, due to the low storage temperature that affect also the growth kinetics of the control. In fact, no differences among samples were detected (Figure 4.7).

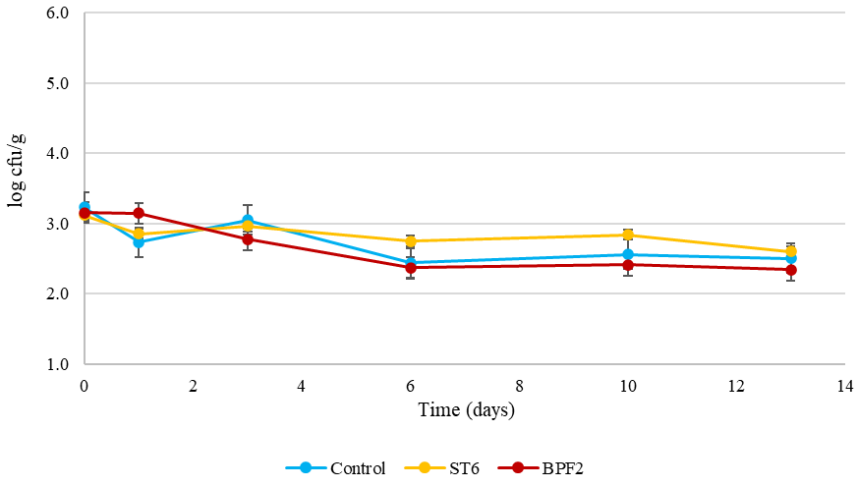


Figure 4.7: *S. enterica* serovar Enteritidis 155 cell counts (log cfu/g) during storage of fresh sausages at 6°C in the challenge test trial, in which LAB strains were inoculated at a final concentration of 6 log cfu/g. Standard deviation is also reported.

However, based on these results, in a second challenge test a higher inoculum of LAB strains were used (approx. 8 log cfu/g). In fact, the low storage temperature allowed a scarce LAB growth, and the strain metabolism did not affect the organoleptic and textural features of the sausages. In this case, no LAB growth was observed. Their initial concentration was 8.50 log cfu/g and 8.64 log cfu/g, respectively for ST6 and BPF2, that at the end of incubation reached value of 8.46 log cfu/g and 8.79 log cfu/g. On the other hand, in the control sample lactobacilli were detected at an initial concentration of 2.75 log cfu/g, that increase up to 7.47 log cfu/g (Figure 4.8).

Even in the presence of these higher LAB concentrations, at the end of the storage no organoleptic impact caused by the increase in LAB concentration was detected, rendering them suitable candidates in bio-protection. It is known that to applicate selected LAB strains as bio-protective cultures, they have to slightly affect or change the desired organoleptic characteristics in fresh products.

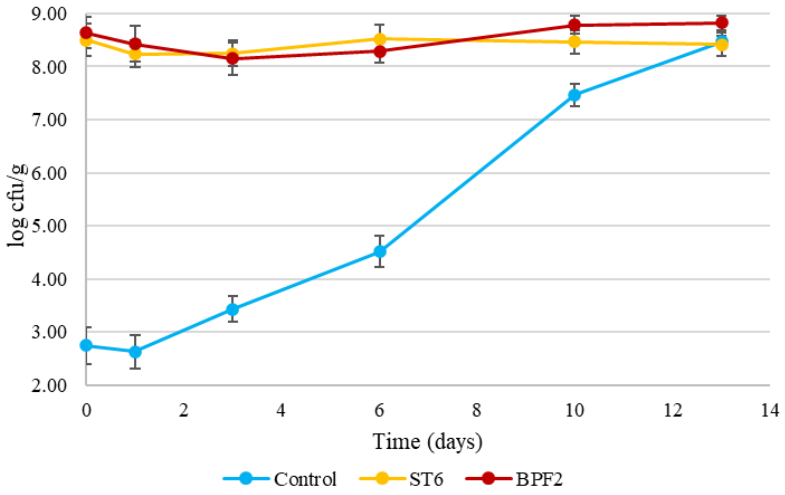


Figure 4.8: LAB microbial counts (log cfu/g) during the storage of fresh sausages at 6°C, monitored in the second challenge test trial (initial inoculum of 8 log cfu/g). Standard deviation is also reported.

Differently from that previously demonstrated, an inhibition effect on the *List. monocytogenes* growth was observed (Figure 4.9). In fact, the target pathogen, that was inoculated at an initial concentration of about 3.5 log cfu/g, after 3 days grown until reaching value of 6.57 log cfu/g at 13 days of storage at 6°C. Conversely, in samples in which LAB cultures were added *Listeria* growth was affected by an initial decrease (after 3 days), followed by a small increase that led to achieve a final maximum cell load of 4.60 log cfu/g and 3.30 log cfu/g in the presence of ST6 and BPF2, respectively.

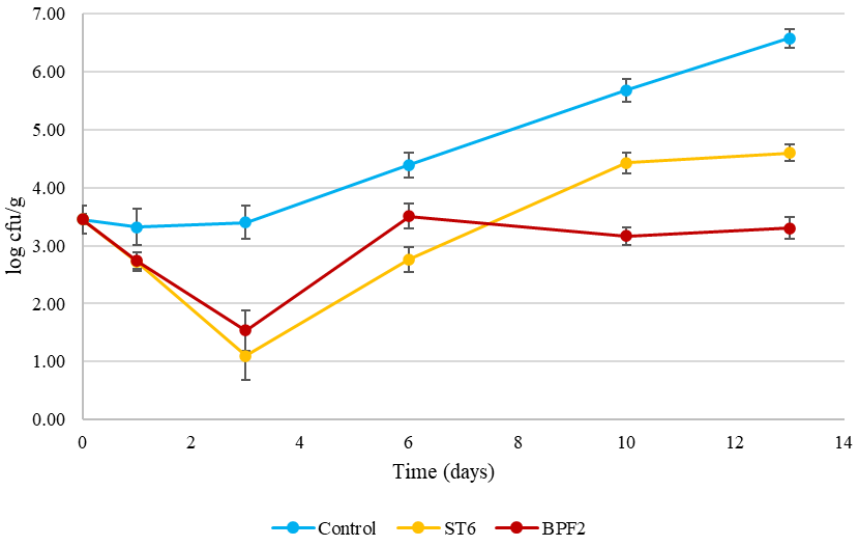


Figure 4.9: *Listeria monocytogenes* ScottA cell counts (log cfu/g) during storage of fresh sausages at 6°C in the challenge test trial, in which LAB strains were inoculated at a final concentration of 8 log cfu/g. Standard deviation is also reported.

On the other hand, despite the higher initial LAB concentrations, bio-protective candidate strains were not able to impact on the behaviour of *S. enterica* serovar Enteritidis growth (Figure 4.10).

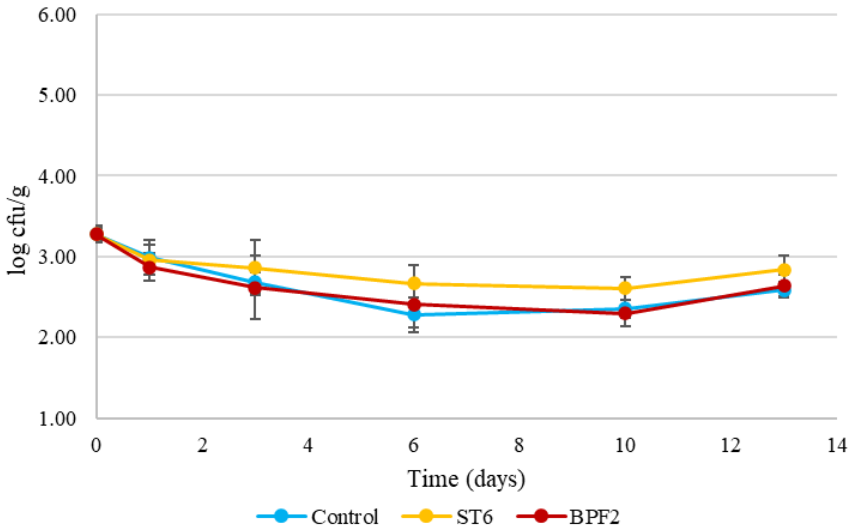


Figure 4.10: *S. enterica* serovar Enteritidis 155 cell counts (log cfu/g) during storage of fresh sausages at 6°C in the challenge test trial, in which LAB strains were inoculated at a final concentration of 8 log cfu/g. Standard deviation is also reported.

4.1.4 Conclusions

The shelf-life of fresh sausages is limited mainly by their high a_w (0.970) and the high pH (approx. 6) and it is strictly dependent on the maintenance of the cold chain (0-4°C). Even small increase of temperature can speed up the spoilage process, bringing to changes in odour, colour and texture and production of exudates. The speed and intensity of this degradation is linked to the quantitative and qualitative composition of initial microbiota and can also allow the growth of pathogenic species, in particular *List. monocytogenes* and *Salmonella* spp. Even though this kind of products must be cooked, the presence of *Listeria* can be a concern and in Italy some regional rules suggested a maximum concentration of 1000 cfu/g before cooking (Direzione Sanità della Regione Piemonte, 2011).

The results of this study demonstrated a strong *in vitro* anti-listerial activity by *P. acidilactici* ST6 and *Ltp. paraplantarum* BPF2 strains, which were able to counteract the growth of *List. monocytogenes* ScottA. In particular, ST6 induced an initial pathogen counts decrease followed by a small recovery of its growth, while BPF2 strain showed a bacteriostatic effect during the entire co-inoculating period in broth culture. On the other hand, no growth inhibition was observed against *S. enterica* serovar Enteritidis 155.

Differently from *in vitro* tests, *P. acidilactici* ST6 and *Ltp. paraplantarum* BPF2 failed to exert their potential anti-listerial activity when applied in *in vivo* tests, in which these strains were inoculated at an initial concentration of about 6 log cfu/g in fresh sausages. This could be attributed to the low temperature applied. On the other hand, successive trials characterised by a higher LAB inoculum concentration (approx. 8 log cfu/g) showed a promising inhibition activity against *List. monocytogenes*.

However, an optimisation of the preculture and condition of inoculum could further favour bacteriocins production and improve their bio-protective use for this purpose.

Work under submission as:

Tabanelli, G., **Barbieri, F.***, García-López, D., Baños, A., Garcia Madero, J.M., Bassi, D., Gardini, F., Montanari, C. Application of autochthonous LAB as bio-protective cultures in fresh sausages. Under preparation to be submitted to *Food Control*.

4.2 Use of two anti-listerial autochthonous starter strains to produce Spanish fermented sausages (salchichónes)

4.2.1 Introduction

Fermented meat sausages are produced using lean meat and fat added with salt spices and other additives, such as nitrates, nitrites, ascorbate and sugar. These products are then stuffed in natural or artificial casings and subjected to a fermentation and drying process (ripening), which brings to modification in parameters such as pH and a_w, that allow to obtain a final product with the desired organoleptic and sensorial characteristics and sausage safety. The microbial activity is guided throughout fermentation and ripening by lactic acid bacteria (LAB) and coagulase negative staphylococci (CNS) (in some cases micrococci). Moreover, in Mediterranean tradition, fungi are also used, among which moulds and, eventually, yeasts (García-Díez and Saraiva, 2021; Laranjo et al., 2019).

LAB play their primary role in the first days after manufacturing, contributing to lower the pH by organic acids production (mainly lactate), which is relevant to reduce the growth potential of undesired bacteria (spoilers or pathogens) and to reach the isoelectric point of meat protein, thus favouring the water evaporation and textural properties acquisition (Cocconcelli and Fontana, 2014). On the other side, staphylococci contribute to flavour formation and nitrate reduction, while moulds are necessary to establish the correct dehydration conditions and concur to flavour formation (Berni, 2015; Carballo, 2012; Cocconcelli and Fontana, 2014).

The first indications concerning the possibility to use selected starter cultures to guide the fermentation of salamis go back to the Fifties of the last centuries (Niinivaara 1955, 1964) and, nowadays, their use is common in industrial productions (Laranjo et al., 2019).

Regarding the selection of LAB starter cultures, technological, safety and quality criteria are considered (Laulund et al., 2017). Among the first, the most important are described by the acidification rate, through the homofermentative pathway, the growth in the presence of high NaCl concentration, the resistance to nitrate and nitrite, good growth performances at the temperature adopted in the manufacturing protocols and the ability to survive and multiply under the nutritional stressing conditions, that characterised fermented sausages during ripening. The safety issues mainly consist in the inability to produce biogenic amines (BA) and the absence of antibiotic-resistance horizontally transmissible (Cocconcelli and Fontana, 2014; Laranjo et al., 2019; Talon and Leroy, 2011). Finally, the main quality parameter considered are the enzymatic activity (lipases and proteases) and their contribution to aroma profile formation (Carballo, 2012).

Recently, another important character is considered to select starter cultures. This consists in the bio-protective antimicrobial activity against pathogenic species, such as *Salmonella* spp., *List. monocytogenes*, *E. coli* ETEC or *Staph. aureus*. This bio-protective aspect depends not only on the higher competition of selected cultures expressed in the environment, but also on their possibility to produce specific molecules characterised by bacteriostatic or bactericidal effects, such as bacteriocins, reuterin (3-hydroxypropionaldheyde) and other antimicrobial peptides (Laranjo et al., 2019; Pérez-Ramos et al., 2021; Simons et al., 2020; Woraprayote et al., 2016; Yap et al., 2021). In the last years an increasing trend of outbreaks linked to the consumption of fermented sausages have been signalled (Holck et al., 2017; Omer et al., 2018). This increase is related to several factors, among which the general trend in NaCl reduction, the diminution or elimination of nitrate and nitrite salts and not correct procedures in fermentation and drying processes are the most important.

Despite of the great diffusion of starter cultures in fermented sausage industry, in Europe there are still several traditional and artisanal products obtained through spontaneous fermentations (Chapter 3.1). These productions deserve as an important biodiversity reservoir of strains to be exploited as new potential starter cultures (Franciosa et al., 2021). These autochthonous strains could favour the local differentiation of traditional products, affected by the standardization due to the relative low number of selected cultures available, and could improve the safety of the products depending on their bio-protective features.

During the previous studies performed in this thesis, the biodiversity of LAB in spontaneously fermented meat has been characterised (Chapter 3.2 and 3.3) and some applications of selected strains have been assessed (Chapter 4.1). Based on the obtained results, the attention was focused on 2 LAB strains isolated from Spanish fermented sausages with a promising anti-listerial activity due to the synthesis of bacteriocin (pediocin and plantaricin): *Lactiplantibacillus paraplantarum* BPF2 and *Pediococcus acidilactici* ST6 (Chapter 4.1).

In this part of the PhD thesis, *Ltp. paraplantarum* BPF2 and *P. acidilactici* ST6 were tested as starter cultures to produce Spanish fermented sausages (salchichón) in pilot plants. Moreover, the mixture of these 2 strains was evaluated and a sample added with a commercial starter culture (RAP) and a control sample (obtained without any starter culture) were also produced. The sausages fermentation was monitored by analysing physico-chemical parameters (pH and weight loss), microbial counts, aroma profiles of the finished products and BA content. Finally, a challenge test was performed against *List. monocytogenes*.

4.2.2 Materials and methods

4.2.2.1 LAB strains and growth conditions

Ltp. paraplantarum BPF2 and *P. acidilactici* ST6, were isolated from Spanish spontaneously fermented sausages. The strains were stored in 20% (w/v) glycerol at -80°C and pre-cultivated for 24 h at 30°C in MRS broth (Oxoid).

4.2.2.2 Salchichón production

The fermented sausages used in these analyses were produced by DOMCA (Granada, Spain). They were prepared with lean pork meat (75%) and pork fat (25%) and a total of 20 kg of meat batter were minced in an 8 mm hole plate grinder (Braher International, Donostia, Spain) and mixed (Mainca, Barcelona, Spain) for 5 min with salt (2.3%), dextrose (0.5%) and a commercial mixture of spices and preservatives, including nitrate and nitrite (180 mg/kg) (Preparado salchichón completo S/A Ref 110347, DOMCA, Granada, Spain).

The mixture was divided into 5 batches (approx. 4 kg) into which different LAB cultures were inoculated at a final concentration of about 6.3 log cfu/g. In particular, *Ltp. paraplantarum* BPF2, *P. acidilactici* ST6 singularly and the mixture of these 2 LAB strains (MIX) were used. In addition, a fermented sausage obtained with a commercial starter culture, containing a mixture of *Lat. sakei*, *Ltp. paraplantarum*, *Staph. xylosus* and *Staph. carnosus* (RAP; Biovitec, France), and a control, without any LAB culture added, produced in the same condition of the other samples, were considered.

After that, each batch was split and stuffed separately using a hydraulic stuffer (EC-12, Mainca SL) into natural lamb casings of 50 mm calibre (Villena, Granada, Spain). Between the stuffing of each batch, cleaning was carried out with a 1% bleach solution and subsequently rinsed, to avoid cross-contamination with the different microorganisms. The ripening process for each sample was carried out at 18ºC for 10 h, then the samples were maintained at 24°C for 48 h. Finally, products were dried in a chamber with 80% humidity and a temperature of 14°C for 28 days.

The obtained samples were monitored and evaluated during all the production process. The trial was performed in triplicate (three independent sausages for each production).

4.2.2.3 Physico-chemical and microbial analyses

Each sample was monitored during ripening regarding physico-chemical parameters and microbiological aspects. In particular, pH was measured by using a pH-meter Basic 20 (Crison Instruments, Barcelona, Spain) during fermentation and ripening processes. Moreover, at each sampling time, sausages were also weighed to calculate the mean weight loss (%) with respect to

the initial one. In addition, the principal microbial groups were detected through a microbiological sampling (Paragraph 3.1.2.3). Lactic acid bacteria (LAB), staphylococci, *Enterobacteriaceae* and yeasts were detected.

All these analyses were performed in triplicate (three different sausages) and the results were expressed as mean value.

4.2.2.4 Evaluation of the aroma profile in the finished products

Gas-chromatography-mass spectrometry coupled with the solid-phase microextraction (GC-MS-SPME) technique was employed for the volatile organic compounds (VOCs) analysis of the products at the end of ripening, following the procedure previously described (Paragraph 3.1.2.6).

An Agilent Hewlett Packard 7890 GC gas-chromatograph equipped with a MS detector 5975 MSD (Hewlett-Packard) were used to peaks detection, while volatile peaks identification was carried out using Agilent Hewlett-Packard NIST 2011 mass spectral library (NIST, 2011) and the data were expressed as the ratio between each molecules peak area and the peak area of internal standard. All the analyses were performed in triplicate.

4.2.2.5 Quantification of biogenic amines content

Biogenic amines quantification was determined for each product, collected at the end of the ripening, after an extraction with trichloroacetic acid (TCA) 5%. Before the HPLC injection, the extracts were subjected to a dansyl-chloride derivatization (Sigma-Aldrich), according to Pasini et al. (2018). As previously reported in Paragraph 3.1.2.5, an HPLC Agilent Technologies 1260 Infinity with the automatic injector (G1329B ALS 1260, loop of 20 µl), equipped with a UV detector (G1314F VWD 1260) set at 254 nm, was used to detect the presence of the principal BA (*i.e.* histidine, tyramine, putrescine, cadaverine and 2-phenylethylamine).

The BA amount was expressed as mg/kg with reference to a calibration curve obtained through aqueous dansyl-chloride-derivatized BA standards of concentrations ranging from 10 to 200 mg/l (Sigma-Aldrich). The detection limit for all these compounds was 3 mg/kg of the sample under the adopted conditions. All the analyses were performed in triplicate.

4.2.2.6 Challenge test against *Listeria monocytogenes*

After the characterisation of the fermented sausages obtained through the application of different starter cultures, a challenge test against *List. monocytogenes* was performed, in order to evaluate the anti-listerial activity of each tested strain. The antimicrobial effect was assessed through the inoculum of the target microorganism into the meat batter before stuffing at a final

concentration of about 3 log cfu/g. *List. monocytogenes* growth behaviour was monitored over time by sampling onto LSO plates (Oxoid), following the procedure explained in the Paragraph 3.1.2.3. Data were collected after 48 h of incubation at 37°C.

4.2.2.7 Statistical analyses

Data collected were statistically analysed through one-way ANOVA procedure of Statistica 8.0 software (StatSoft Inc.) and significant differences between the conditions were evaluated with the LDS test ($p \leq 0.05$).

4.2.3 Results and discussion

4.2.3.1 Physico-chemical and microbial characterisation of Spanish fermented sausages

During these analyses, 5 batches of salchichón characterised by different starter cultures were monitored over time for controlling the pH dynamics and the weight losses (Table 4.6 and Table 4.7). Results concerning pH showed a marked decrease after 7 days (approx. 0.8 unit). In general, the sample RAP showed significantly lower values after the fermentation, while no relevant difference was observed at the end of ripening, after which the final pH value ranged between 4.9 and 5.1.

Table 4.6: pH dynamics monitored during the production of salchichón, obtained with different LAB starter cultures. Standard deviations are also reported. Moreover, as a result of ANOVA test, different letters indicate significant differences among pH values of each sample at each time of analysis.

pH	0 days	7 days	15 days	22 days	30 days
Control	6.34 ± 0.03	5.63 ± 0.03^{a}	5.44 ± 0.02^{a}	5.33 ± 0.03^{ac}	5.13 ± 0.02^{a}
RAP	6.29 ± 0.05	5.46 ± 0.04^{b}	5.39 ± 0.01^{b}	5.20 ± 0.04^{b}	5.03 ± 0.03^{b}
BPF2	6.32 ± 0.03	5.57 ± 0.03^{c}	5.42 ± 0.01^{a}	5.18 ± 0.02^{b}	5.08 ± 0.01^{b}
ST6	6.37 ± 0.04	5.70 ± 0.01^{d}	5.51 ± 0.01^{c}	5.35 ± 0.04^{c}	5.05 ± 0.04^{b}
MIX	6.35 ± 0.04	5.65 ± 0.02^{a}	5.43 ± 0.03^{a}	5.28 ± 0.04^{a}	5.12 ± 0.03^{a}

Concerning weight losses, the control was characterised by the slower kinetics, reaching the lowest final value (24.61%), compared with decreases observed in fermented sausages with starter cultures (28.88% and 31.07%).

Table 4.7: Weight losses measured during the production of salchichón, obtained with different LAB starter cultures. Standard deviations are also reported. Moreover, as a result of ANOVA test, different letters indicate significant differences among weight loss percentages of each sample at each time of analysis.

Weight loss (%)	0 days	7 days	15 days	22 days	30 days
Control	0.00	6.49 ± 0.64^{a}	11.55 ± 1.12^{a}	18.73 ± 0.21^{a}	24.61 ± 0.89^{a}
RAP	0.00	7.38 ± 0.33^{b}	21.13 ± 0.76^{b}	23.88 ± 2.54^{b}	28.88 ± 2.35^{b}
BPF2	0.00	6.74 ± 0.41^{a}	19.37 ± 2.47^{bc}	22.74 ± 2.83^{b}	31.07 ± 2.92^{b}
ST6	0.00	9.33 ± 1.23^{c}	17.75 ± 2.83^{c}	21.75 ± 1.82^{b}	29.06 ± 2.91^{b}
MIX	0.00	9.69 ± 1.66^{c}	21.31 ± 0.97^{b}	24.69 ± 2.78^{b}	29.86 ± 3.81^{b}

Regarding microbial characterisation, the results are shown in Table 4.8. In the control, the initial LAB count on MRS was 3.30 log cfu/g. This number gradually increased during ripening. However, the final concentration of this group did not exceed a final value of 6.30 log cfu/g. The addition of LAB starter cultures determined initial LAB concentration comprised between 6.24 and 6.41 log cfu/g. After 7 days this microbial group increased up to 8 log cfu/g and more in all the sample and reached, after 30 days, counts higher than 9 log cfu/g independently on the cultures added.

Staphylococci were present at a concentration of 3.70 log cfu/g and only in the samples RAP, added with a commercial starter culture containing *Staph. xylosus* and *Staph. carnosus*, the initial value was 6.62 log cfu/g. In this latter case, the concentration of this microbial group was higher than 8 log cfu/g at the end of ripening. In the other samples they increased up to values ranging from 5.11 to 5.30 log cfu/g, without significant difference.

Concerning *Enterobacteriaceae*, the initial contamination (3.58 log cfu/g) did not show relevant increases during ripening, with final values ranging from 3.83 and 3.89 log cfu/g, without differences in relation to the different starter cultures used. Finally, yeasts, characterised by an initial value of 2.30 log cfu/g, increased in all the samples up to 6 log cfu/g and more, but the higher counts were attained in the control samples and in the sausages inoculated with RAP. These high counts of yeasts at the end of ripening were consistent with other similar Spanish fermented sausages (Flores et al., 2015).

The initial microbial quality of the meat mixture was acceptable, as demonstrated by the low *Enterobacteriaceae* counts and by the low concentration of the other microbiological groups monitored. In the control sample the low concentration of LAB on MRS, coupled with a pH decrease comparable to fermented sausages containing starter cultures, could be explained by the presence of other bacteria, such as carnobacteria. They cannot grow on MRS containing acetate, even if present in spontaneously fermented meat products (Van Reckem et al., 2021; Geeraerts et al., 2018).

Table 4.8: Concentrations (log cfu/g) of the main microbial groups in the 5 analysed fermented sausages during the production. Standard deviations are also reported. Moreover, as a result of ANOVA test, different letters indicate significant differences among microbial counts of each sample at each time of analysis.

Microbial groups	Time (days)	Control	RAP	BPF2	ST6	MIX
Lactobacilli	**0**	3.30 ± 0.15^{a}	6.41 ± 0.09^{b}	6.29 ± 0.07^{b}	6.24 ± 0.12^{b}	6.37 ± 0.08^{b}
	1	3.40 ± 0.14^{a}	7.28 ± 0.10^{b}	7.32 ± 0.09^{b}	7.59 ± 0.08^{b}	7.24 ± 0.07^{b}
	7	4.53 ± 0.07^{a}	8.15 ± 0.07^{bc}	8.28 ± 0.10^{c}	7.93 ± 0.07^{b}	8.01 ± 0.10^{b}
	15	5.80 ± 0.10^{a}	8.22 ± 0.08^{b}	8.60 ± 0.07^{c}	8.09 ± 0.08^{b}	8.15 ± 0.08^{b}
	22	5.92 ± 0.07^{a}	8.84 ± 0.07^{b}	8.37 ± 0.08^{c}	8.05 ± 0.09^{d}	8.93 ± 0.07^{b}
	30	6.29 ± 0.09^{a}	9.39 ± 0.07^{b}	9.30 ± 0.07^{b}	9.05 ± 0.07^{c}	9.30 ± 0.07^{b}
Staphylococci	**0**	3.70 ± 0.09^{a}	6.62 ± 0.07^{b}	3.70 ± 0.13^{a}	3.70 ± 0.12^{a}	3.70 ± 0.15^{a}
	1	3.70 ± 0.08^{a}	6.72 ± 0.09^{b}	3.90 ± 0.14^{c}	3.80 ± 0.11^{c}	3.90 ± 0.11^{c}
	7	3.99 ± 0.10^{a}	6.93 ± 0.09^{b}	3.95 ± 0.15^{a}	3.95 ± 0.13^{a}	3.98 ± 0.09^{a}
	15	4.42 ± 0.11^{a}	7.20 ± 0.07^{b}	4.71 ± 0.08^{a}	4.68 ± 0.08^{a}	4.70 ± 0.07^{a}
	22	4.90 ± 0.07^{a}	7.91 ± 0.07^{b}	4.95 ± 0.09^{a}	4.93 ± 0.07^{a}	4.87 ± 0.08^{a}
	30	5.11 ± 0.08^{a}	8.45 ± 0.08^{b}	5.30 ± 0.12^{c}	5.22 ± 0.09^{c}	5.15 ± 0.10^{a}
Enterobacteria	**0**	3.58 ± 0.20	3.58 ± 0.12	3.58 ± 0.19	3.58 ± 0.16	3.58 ± 0.12
	1	3.67 ± 0.18	3.66 ± 0.11	3.63 ± 0.17	3.68 ± 0.13	3.60 ± 0.11
	7	3.82 ± 0.16	3.70 ± 0.16	3.79 ± 0.10	3.75 ± 0.16	3.75 ± 0.15
	15	3.85 ± 0.10	3.82 ± 0.10	3.85 ± 0.20	3.83 ± 0.14	3.84 ± 0.12
	22	3.86 ± 0.10	3.84 ± 0.11	3.87 ± 0.11	3.83 ± 0.14	3.86 ± 0.12
	30	3.87 ± 0.12	3.85 ± 0.11	3.88 ± 0.12	3.83 ± 0.13	3.89 ± 0.11
Yeasts	**0**	2.30 ± 0.10	2.30 ± 0.17	2.30 ± 0.20	2.30 ± 0.18	2.30 ± 0.19
	1	2.49 ± 0.18	2.52 ± 0.14	2.53 ± 0.16	2.55 ± 0.16	2.49 ± 0.15
	7	4.54 ± 0.10	4.55 ± 0.01	4.47 ± 0.12	4.37 ± 0.14	4.36 ± 0.08
	15	5.90 ± 0.14^{a}	5.67 ± 0.09^{ab}	5.53 ± 0.13^{b}	5.17 ± 0.14^{c}	5.50 ± 0.07^{b}
	22	5.91 ± 0.11^{a}	5.70 ± 0.10^{b}	5.64 ± 0.12^{b}	5.48 ± 0.13^{c}	5.67 ± 0.09^{b}
	30	6.77 ± 0.07^{a}	6.81 ± 0.07^{a}	6.37 ± 0.07^{b}	6.01 ± 0.09^{c}	5.95 ± 0.07^{c}

4.2.3.2 Detection of biogenic amines content

Each sample was analysed also regarding the BA content at the end of ripening by using HPLC technique. Data reported in Table 4.9 showed that histamine and 2-phenylethylamine were always under the detection limit in all the samples. However, relevant differences were observed for other amines.

In particular, tyramine reached its maximum concentration (150.9 mg/kg) in the control, obtained without the addition of starter cultures. A reduced concentration of this BA was detected in samples with the commercial starter RAP and *P. acidilactici* ST6 (approx. 120 mg/kg), while the presence of BPF2 and the combination BPF2+ST6 (MIX) determined the lower accumulation of tyramine. Cadaverine, detected at a concentration of 245.1 mg/kg in the spontaneously fermented samples, was more than halved in the fermented sausages containing ST6 and BPF2 (with concentrations ranging from 95.6 to 114.7 mg/kg), while RAP further reduced this level, reaching a

value of 58.5 mg/kg. The most relevant effect concerned putrescine. In fact, this BA was detected at 143.5 mg/kg in the control, while in the other sausages its level was very low, ranging from 8.3 mg/kg (RAP) and 25.3 (BPF2) mg/kg.

The total BA content was more than double in the sausages produced without starter culture (approx. 540 mg/kg), confirming the positive effect that starter cultures can exert on the accumulation of these compounds (Barbieri et al., 2019; Laranjo et al 2019). The most relevant effects interested putrescine and cadaverine. Putrescine derives from the decarboxylation of ornithine, which can be produced both by Gram-negative and Gram-positive bacteria through different pathways. In any case, arginine plays a crucial role in the synthesis of this BA (Wunderlichová et al, 2014). The production of cadaverine is often attributed to Gram-negative bacteria. However, the presence of lysine decarboxylase activity has been demonstrated in lactobacilli (Berthoud et al., 2022; Pessione et al., 2005; Romano et al., 2013), explaining the high presence of this BA in fermented sausages, even if characterised by a low concentration of *Enterobacteriaceae*.

Table 4.9: Concentrations (mg/kg) of the main biogenic amines detected in the 5 analyses fermented sausages at the end of ripening. Standard deviations are also reported. Moreover, as a result of ANOVA test, different letters indicate significant differences among the content of BA in each sample.

Samples	Histamine	Tyramine	Putrescine	Cadaverine	2-phenylethylamine	Tot
Control	-*	150.92 ± 1.10^{a}	143.48 ± 1.63^{a}	245.06 ± 2.40^{a}	-	**539.46^{a}**
RAP	-	122.37 ± 0.89^{b}	8.31 ± 0.29^{b}	58.51 ± 0.57^{b}	-	**189.20^{b}**
BPF2	-	93.10 ± 0.68^{c}	25.28 ± 0.29^{c}	101.45 ± 0.99^{c}	-	**219.84^{c}**
ST6	-	117.85 ± 0.86^{b}	19.98 ± 0.23^{c}	114.65 ± 1.12^{c}	-	**252.48^{d}**
MIX	-	100.64 ± 0.73^{b}	14.16 ± 0.16^{c}	95.56 ± 0.94^{c}	-	**210.36^{c}**

*: under the detection limits (3 mg/kg)

4.2.3.3 Aroma profile of finished Spanish fermented products

The volatile profile of the different fermented sausages at the end of ripening is reported in Table 4.10, in which the molecules detected are grouped according to their chemical structure. The total amount of each chemical class is showed in Figure 4.11.

Aldehydes were present at higher level in the control and in the sausages obtained with the commercial starter culture (RAP). The difference was mainly linked to hexanal (10.12 and 19.73, respectively). This aliphatic aldehyde is the main product of fatty acid autoxidation and derived from linoleic acid (Ordóñez et al., 1999).

Among ketones, acetone, detected in negligible amount in the control, was present at high level in the samples RAP and ST6, while its presence was more relevant in the samples BPF2 and MIX (BPF2+ST6). Diacetyl (2,3-butanedione) reached its maximum values in the samples ST6 and

MIX and it is not surprising that acetoin (3-hydroxy-2-butanone), which derive from diacetyl reduction, was higher in the same samples. All these molecules derive from pyruvate metabolisms and indicate different activations of the pathways involving this organic acid (Barbieri et al., 2020; Carballo, 2012). In addition, 2-pentanone and 2-nonanone were more abundant in the control and in RAP, indicating the possible involvement of yeasts, whose concentration was higher in these samples, in the production of these methyl ketones through β-oxidation of fatty acids.

Ethanol was detected in highest amount in the control, but also the sample ST6 was characterised by high concentration of this alcohol, which showed a low level in RAP. Interestingly, ethanol is one of the end products of pyruvate pathways (Axelsson, 2004). Among alcohols, hexanol, deriving from hexanal reduction, confirmed the previous observations concerning aldehydes: the use of the new selected strains (ST6 and BPF2) allowed the reduction of the presence of products resulting from lipid autoxidation. Similar results were observed for other aliphatic alcohols, such as pentanol and 1-octen-3-ol.

Acetic acid presence resulted higher in the RAP samples, while no relevant differences were observed among the other sausages. Hexanoic (caproic) acid was present at higher proportion in the control and in RAP samples, if compared with the selected strain. Similar results were observed also for pentanoic acid and 4-methyl-pentanoic acid. The origin of hexanoic acid may be attributed to the activity of yeasts, that reached their maximum concentration in these two samples. In fact, it is well known that yeasts can produce short or medium chain fatty acids (Gajewski et al., 2017; Hu et al., 2018). Under these conditions, it is not surprising that ethyl hexanoate (caproate) was the major ester produced in these two samples (control and RAP), while ethyl acetate prevailed in the other fermented sausages.

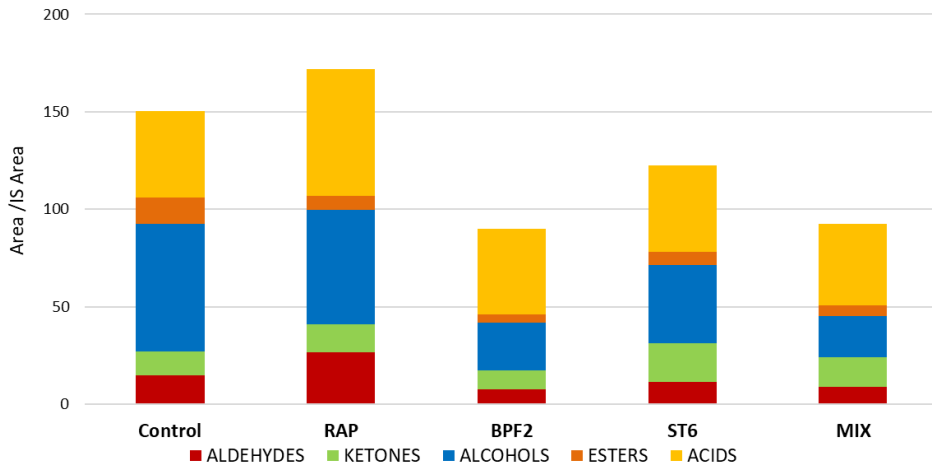


Figure 4.11: Presence of the different classes of volatile organic compounds (VOCs) in the samples. The values are expressed as the ratio between the peak area of the compound considered and the area of the internal standard.

Table 4.10: Volatile organic compounds (VOCs) detected by SPME-GC-MS in the samples, expressed as ratio between peak area of each molecule and peak area of the internal standard (4-methyl-2-pentanol). The standard deviation was always below 5%.

Volatile compounds	Control	RAP	BPF2	ST6	MIX
Butanal	-*	0.18	-	0.40	-
Hexanal	10.12	19.73	3.32	4.69	2.62
Nonanal	1.43	2.41	2.12	1.40	3.64
Decanal	0.19	0.35	0.30	0.88	0.46
Benzaldehyde	0.70	2.22	0.74	1.34	0.74
Benzene acetaldehyde	2.24	1.54	0.94	2.73	1.29
Aldehydes	**14.69**	**26.44**	**7.42**	**11.45**	**8.75**
Acetone	0.80	9.81	5.66	11.68	1.66
2-butanone	0.32	0.76	0.52	0.94	0.64
2,3-butanedione	-	0.32	-	1.44	1.26
2-pentanone	3.61	2.38	1.17	1.40	0.78
Methyl isobutyl ketone	0.38	2.22	1.64	3.02	0.65
3-hexen-2-one	2.68	3.78	2.71	2.50	1.91
2-octanone	1.30	0.97	0.45	0.56	0.28
2-butanone-3-hydroxy	2.20	2.25	2.99	9.43	9.93
2-nonanone	1.93	1.68	0.24	0.54	-
Ketones	**12.42**	**14.36**	**9.73**	**19.83**	**15.45**
Ethyl alcohol	28.41	4.90	8.27	19.81	9.87
1-pentanol	3.42	4.39	1.40	1.36	1.30
2-heptanol	0.68	0.15	-	0.55	-
1-hexanol	22.00	38.86	8.27	4.96	3.09
1-octen-3-ol	1.17	3.94	0.87	0.81	0.84
1-hexanol, 2-ethyl	1.03	1.42	1.20	3.23	1.06
1-octanol	3.02	1.83	0.85	3.00	0.67
Nonanol	1.19	-	0.24	0.89	-
Benzyl alcohol	3.06	2.80	2.33	3.33	2.48
Phenylethyl alcohol	1.36	0.73	1.09	2.09	1.56
Alcohols	**65.36**	**59.03**	**24.53**	**40.02**	**20.88**
Ethyl acetate	4.03	0.80	2.53	2.64	2.78
Butanoic acid, ethyl ester	0.88	-	0.18	0.57	0.22
Hexanoic acid, ethyl ester	6.83	4.98	1.25	2.32	1.99
Acetic acid, hexyl ester	0.32	0.77	-	-	-
Octanoic acid, ethyl ester	0.97	-	-	0.55	0.30
Decanoic acid, ethyl ester	0.42	0.47	0.38	0.73	0.52
Esters	**13.44**	**7.02**	**4.34**	**6.81**	**5.80**
Acetic acid	28.31	41.11	32.10	30.83	28.21
Propanoic acid	0.00	1.40	0.62	0.86	0.71
Butanoic acid, 3-methyl	0.98	1.26	1.24	2.20	1.89
Pentanoic acid	2.34	2.74	1.03	1.66	1.42
Pentanoic acid, 4-methyl	1.09	1.90	0.85	0.52	0.70
Hexanoic acid	9.89	10.87	5.52	4.06	4.65
Heptanoic acid	0.74	1.11	0.64	0.44	0.64
Octanoic acid	-	2.58	-	1.60	1.62
Nonanoic acid	1.13	0.73	0.70	0.86	0.70
n-decanoic acid	-	1.35	1.13	1.37	0.90
Acids	**44.47**	**65.05**	**43.84**	**44.39**	**41.45**

*: not detected under the adopted conditions

4.2.3.4 Challenge test against *Listeria monocytogenes*

With the aim to evaluate the effective bioprotective activity of the different starter cultures, fermented sausages were prepared under the same conditions previously adopted, but the meat mixture was inoculated with *List. monocytogenes* at a concentration of about 3.2 log cfu/g. During

fermentation and ripening, the concentration of the pathogen was monitored, and the results are reported in Figure 4.12.

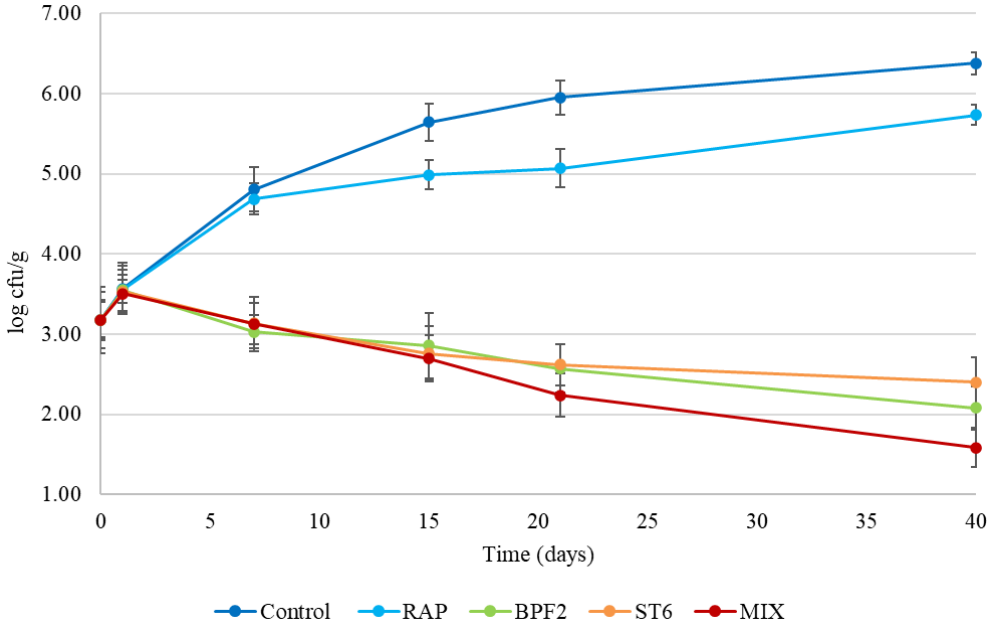


Figure 4.12: *List. monocytogenes* growth kinetics in the challenge test performed on Spanish fermented sausages.

In the control without starter cultures *List. monocytogenes* constantly grew and reached a final concentration of 6.4 log cfu/g. In the RAP samples a similar behaviour was observed, even if after 7 days the concentration was lower, and the final value was 5.7 log cfu/g. A different situation characterised the samples inoculated with BPF2 and ST6 and their mixture. In fact, after a small initial increase (counts of approx. 3.5 log cfu/g after 1 day), the viable counts of *List. monocytogenes* progressively decreased and reached a final concentration of 2.1 log cfu/g and 2.4 log cfu/g in the presence of BPF2 and ST6, respectively, and 1.6 log cfu/g when the two strains were inoculated together.

List. monocytogenes is one of the main safety concerns in the production of Mediterranean salamis (Meloni, 2015). In fact, the thermal profiles used during fermentation and ripening and the reduction of NaCl initial concentration, pursued in the last decades, can favour the growth and survival of this pathogen (Barcenilla et al., 2022b). In addition, the trend to reduce or eliminate the use of nitrate and nitrites can represent a further risk for *Listeria* growth. Different solutions have been proposed to counterbalance this increased risk. Among them, the use of bioprotective cultures plays a crucial role to inhibit listeria growth or favour its death (Holck et al., 2017; Tabanelli et al., 2022). The new strain used in these trials demonstrated a relevant inhibiting activity against *List. monocytogenes*, not only if compared to the spontaneously fermented control, but also if compared with the commercial starter culture RAP.

4.2.4 Conclusions

The use of the two strains, *Ltp paraplantarum* BPF2 and *P. acidilactici* ST6, selected on the basis of their inhibiting properties against *List. monocytogenes*, proved to be extremely effective in counteracting the presence of this pathogen in salami during the challenge test. In fact, the results obtained were extremely positive when compared to both the spontaneously fermented sample (control) and the sausage fermented using the commercial starter culture (RAP). The same strains also showed a good aptitude in reducing the BA accumulation observed in the control. In addition, the technological parameters of fermented sausages during ripening were similar to those observed in the product obtained using RAP. The final products presented comparable behaviour in terms of pH and weight losses over time but presented interesting differences in relation to the volatile profile. This aspect can make the use of these new strains interesting for a sensory differentiation of the final products, increasing the possibility for tailor made fermentation aimed to improve the recognisability and the peculiar traits of fermented sausages.

For these reasons, the anti-listerial activity showed by BPF2 and ST6 is very interesting and needs to be further investigated for the exploitation in industrial processes.

Work under submission as:

García-López, D., **Barbieri, F.***, Baños, A., Garcia Madero, J.M., Gardini, F., Montanari, C., Tabanelli, G. Use of two anti-listerial autochthonous starter strains to produce Spanish fermented sausages (salchichónes). Under preparation to be submitted to *Food Control*.

Chapter 5:

Study of the *in vitro* antimicrobial potential of bio-active compounds against spoilage microorganisms or food-borne pathogens

5.1 Effects of *Rubus fruticosus* and *Juniperus oxycedrus* derivatives on culturability and viability of *Listeria monocytogenes*

5.1.1 Introduction

The request of consumers for safe foods without chemical additives is addressing the recent research towards the replacement of chemical preservatives with natural compounds with a broad spectrum of antimicrobial potential (Prakash et al., 2015). In this perspective, plants can be an important source of molecules that possess strong activities against several microorganisms responsible for food spoilage or that affect their safety.

Derivatives of many aromatic plants were used for centuries in medicine, cosmesis and food production, independently of the real comprehension of their antimicrobial role. These substances are widely accepted by consumers and are generally recognised as safe (GRAS), being derived from edible products. For this reason, many plant extracts, essential oils (EO) or their constituents have been proposed as potential preservative ingredients in several foods or as antimicrobial agents (Vergis et al., 2015).

Mediterranean area is a natural ecosystem characterised by many aromatic plants with these properties (Corona et al., 2017; Mulas and Mulas, 2005). In particular, the *Rosaceae* family are extremely widespread and firstly represented by *Rubus fruticosus*, together with *Rubus ulmifolius*, two plant varieties commonly known as blackberry. Their phenolic extracts (PE) exhibit relevant antioxidant activity, being rich in phenolic acids, flavonoids (anthocyanins, flavonols and tannins), carotenoids and organic acids (Krzepiłko et al., 2021). In addition, these compounds can inhibit the growth of several food-borne pathogens. In their work, Veličković et al. (2021) demonstrated promising antimicrobial activity (especially against *List. monocytogenes*) in different aqueous and acetone extracts of *Rubus discolor*. *R. fruticosus* showed a relevant antimicrobial activity against several microorganisms, including *E. coli*, *Salmonella* Typhi and *Staph. aureus*, while none or scarce effects were observed against yeasts and moulds, as revealed by the minimum inhibitory concentrations (MIC) reported (Riaz et al., 2011). In another study, the growth of *List. monocytogenes*, *E. coli* O157:H7, and *Salmonella* Typhimurium was reduced by the activity of blackberry juice, that also significantly stimulated the growth of the LAB *Ltp. plantarum*, *Ltc. casei* and *Ltc. rhamnosus* (Yang et al., 2014).

Another genus of the Mediterranean maquis is *Juniperus*, belonging to the *Cupressaceae* family. Among the most widespread species, there are *Juniperus communis*, *Juniperus turbinata*, *Juniperus deltoides* and *Juniperus oxycedrus*. *J. oxycedrus* has, for example, traditional history of use in therapeutic and folk medicine for different diseases, such as tuberculosis, pneumonia,

bronchitis, diarrhea, stomach aches, and hyperglycemia (Mrid et al., 2019). Extracts of juniper species have been widely studied in relation to antioxidant activity as well as antimicrobial potential (Boudiba et al., 2021; Dziedzinski, et al., 2020). Many studies are focused on the characterisation of their EO chemical profile. They are constituted by α-pinene, the principal compound, followed by myrcene, sabinene, limonene, germacrene D, δ-cadinene and other terpenes and terpenoids. It is important to consider that the chemical composition varies with seasonal factors, geographical origin, environmental conditions and parts of the plant used for EO production, such as berries or needles (Miceli et al., 2020). The EO of *J. oxycedrus* from Bulgaria showed an antimicrobial potential against Gram-positive bacteria, including *Staph. aureus* and *Ent. faecalis* (Semerdjieva et al., 2020). The same result was obtained by Najar et al. (2020), that evidenced also an antimicrobial effect on *List. monocytogenes*.

Despite their great potential, the application of plant extracts in order to prolong shelf-life and assuring safety of foods is still limited. This is due to the variability of extract composition and conditions adopted in the antimicrobial activity tests, which affect their effects against target microorganisms, as well as to the absence of sufficient knowledge regarding the mode of action (Balouiri et al., 2016). In addition, limitations can derive from their organoleptic and sensorial impact if used at elevated concentrations. Thus, many approaches have been proposed in the framework of hurdle strategy, in which many sub-lethal factors are applied for controlling microbial growth, causing cells death or their inability to multiply.

Given these considerations, in this part of PhD thesis the study was focused onto the characterisation of phenolic extracts and essential oils obtained from Croatian *R. fruticosus* leaves and *J. oxycedrus* needles. These bio-active PE and EO were further tested for their possible antimicrobial effects against *List. monocytogenes* Scott A. The growth dynamics in the presence of sub-lethal concentrations of plant derivatives were modelled to highlight differences in the cell kinetics. In addition, to better evidence their effect on culturability and viability of target microorganism cells, both plate counting and flow cytometric analyses were performed (Davey, 2011; Arioli et al., 2019).

5.1.2 Materials and methods

5.1.2.1 Plant collection and extraction of phenolic extracts (PE) and essential oils (EO)

R. fruticosus leaves and *J. oxycedrus* needles were collected in August of 2020 at the altitude of 420 m (mountain Kozjak, Croatia) and dried in a shady place at room temperature for 7 days. To obtain PE, dried materials were extracted in 50% ethanol through the advanced microwave extraction (MAE) system method (ETHOS X, Milestone Srl, Sorisole, Italy) at 600 W for 5 min.

After that, ethanol was evaporated and the extracts freeze-dried and stored in a cool dark place until analyses (Čagalj et al., 2021).

On the other hand, EO extraction was performed by hydrodistillation, during which 00 g of the dried material was added in a flask with distilled water in Clevenger-type apparatus for 3 h. Pentane and diethyl ether (1:1, v/v) were used for trapping the volatile compounds. The obtained EO was dried over anhydrous sodium sulphate and the obtained extract stored at 4°C in dark vials until analyses (Generalić Mekinić et al., 2021).

5.1.2.2 Characterisation of PE through HPLC analysis

Freeze-dried extracts were dissolved in 50% ethanol and the individual phenolics of PE were identified and quantified through an HPLC Ultimate 3000 (ThermoFisher Scientific) equipped with a UV-Vis DAD (Generalić Mekinić et al., 2014). The sample separation (10 µl injected) was carried out using a Syncronis™ C18 column (250 × 4.6 mm, 5 µm particle size; ThermoFisher Scientific), maintained at 25°C, with a flow of 0.8 ml/min. The run method was characterised by a gradient of three solvents: solvent A (water/formic acid, 98:2, v/v), solvent B (acetonitrile) and solvent C (methanol). The peaks of each phenolics were identified by comparing their retention times and absorption spectra (at two wavelengths 280 nm and 320 nm) with those acquired for corresponding standards. The identified compounds were quantified using external standard calibration curves (injected in five different concentrations). Data were reported as means of three independent analyses. The results were expressed in mg of compound per l of extract (mg/l). All used reagents, solvents, and standards were purchased from Sigma (Sigma-Aldrich), Merck (Darmstadt, Germany), Fluka (Buch, Switzerland), and Kemika (Zagreb, Croatia) and were of adequate analytical grade.

5.1.2.3 Characterisation of EO through GC-MS analysis

The composition of EO obtained from *R. fruticosus* leaves and *J. oxycedru*s needles was determined through a GC-MS (Shimadzu QP2010, Shimadzu, Kyoto, Japan) equipped with an autosampler and a DB-5 60 m × 0.25 mm × 0.25 µm column (Agilent Technologies Italia Spa, Milano, Italy), according to Generalić Mekinić et al. (2021). Before analysis, EO samples were resuspended in hexane and 1 µl was injected into the instrument. The set parameters are listed: injection temperature 260°C, interface temperature 280°C, ion source 220°C, carrier gas (He) with a flow rate 30 cm/s and splitting ratio 1:20. All compounds were identified by comparing their spectra with those reported in NIST 8.0 library (US National Institute of Standards and Technology). For each sample, the results were expressed as a relative percentage of each single peak area with

respect to the total peak area. Only the compounds whose peak area was higher than 0.5% of the total peak area are reported in the final Tables. Data reported are the means of three repetitions.

5.1.2.4 Target microorganism strain and growth conditions

The strain used in these tests was *List. monocytogenes* Scott A, belonging to the collection of the Department of Agricultural and Food Sciences (University of Bologna). The strain was stored in BHI medium (Oxoid) with 20% (w/v) glycerol at -20°C. Before the trials, it was pre-cultivated twice at 37°C for 24 h in BHI medium.

5.1.2.5 Determination of minimum inhibiting concentration (MIC)

The *in vitro* antimicrobial activity of the considered plant derivatives against *List. monocytogenes* was assessed with broth microdilution method using microtiter plates (Corning Incorporated, New York, USA), according to Arioli et al. (2019). For the determination of cell growth/no growth, 198 µl of BHI broth inoculated with *List. monocytogenes* at a final concentration of about 6 log cfu/ml were placed into 200 µl microtiter wells. Plant derivatives were dissolved in ethanol and 2 µl of these solutions were added into each well to obtain final concentrations ranging between 0 and 3 mg/ml. Microtiter plates were incubated at 37°C for 48 h. The MIC results were defined as the lowest concentration of plant derivatives able to prevent a visible microorganism growth in the well.

5.1.2.6 Effect of plant derivatives on *Listeria monocytogenes* growth

The effect of sub-lethal concentrations of plant derivates against *List. monocytogenes* Scott A growth was assessed through the inoculum of the target microorganism into BHI broth in the presence of an amount corresponding to 50% of MIC for each extract (Arioli et al., 2019). The target microorganism was inoculated at a final concentration of about 6 log cfu/ml and the samples were incubated at 20°C and monitored for 144 h. In particular, for each condition 1 mg/ml of plant derivates was added, with the exception of *R. fruticosus* EO, whose concentration was 0.75 mg/ml. At defined times, samples were collected to assess culturability (by plate counting) and viability (by flow cytometry). For sampling (Paragraph 3.1.2.3), appropriate decimal dilutions were plated onto BHI agar medium and incubated at 37°C for 48 h.

Data obtained (plate counts) were modelled with the Gompertz equation (Zwietering et al., 1990):

$$y = k + A \cdot e^{-e^{\left[\left(\frac{\mu_{max} \cdot e}{A}\right) \cdot (\lambda - t) + 1\right]}}$$

where y is the cellular concentration at time t, k is a constant representing the initial cellular load, *A* parameter represents the difference between maximum cellular load reached and the initial cell concentration, μ_{max} is the maximum log cfu/ml increase rate in exponential phase and the parameter λ is the lag phase duration (hours).

Moreover, since in some samples an initial decrease of cell counts was observed, a double-peaked Gompertz equation was used to fit also the first part of the curves (Tattershall et al., 2021).

$$y = k + \left(A_1 \cdot e^{-e^{\left[\left(\frac{\mu_{max1} \cdot e}{A_1}\right)\cdot(\lambda_1 - t)+1\right]}} \right) + \left(A_2 \cdot e^{-e^{\left[\left(\frac{\mu_{max2} \cdot e}{A_2}\right)\cdot(\lambda_2 - t)+1\right]}} \right)$$

In these case, y is the cellular load at time t, k is a constant representing the initial cellular load, *A*1 represents the difference between the initial inoculum and the cellular load reached in the first phase of decrease, *A*2 represent the difference between the minimum cellular load after the first phase of decrease and the maximum cellular load reached in the second growth phase, $\mu_{max}1$ and $\mu_{max}2$ are the maximum log cfu/ml decrease or increase rate in exponential phase, respectively, and the parameters $\lambda 1$ and $\lambda 2$ are the lag phase duration of the two phases.

In both cases, k was maintained constant at 6.30 log cfu/ml as determined by plate counting immediately after the inoculum.

Data modelling was performed using Statistica 8.0 software (Statsoft Inc.).

5.1.2.7 Flow cytometry analysis

Flow cytometry analysis (Accuri C6; BD Biosciences, Milan, Italy) was performed on cell suspension samples collected after 24, 48, and 72 h of incubation in the presence of the different plant derivatives (Arioli et al., 2019).

Before analyses, samples were diluted (if needed) in filtered PBS and the cells were stained with SYBR-Green I (1×) and propidium iodide (PI) 7.5 µM at 37°C for 15 min, in order to let the dye react with the cell. This dual staining allowed to distinguish three sub-populations corresponding to different physiological states: live, injured and dead cells. The data obtained were analysed using the BD ACCURITM C6 software version 1.0 (BD Biosciences).

5.1.3 Results and discussion

5.1.3.1 Characterisation of plant derivatives

The major phenolic compounds of *J. oxycedrus* needles and *R. fruticosus* leaves are reported in Table 5.1.

Table 5.1: Composition of the phenolic extracts (PE) of *Juniperus oxycedrus* needles and *Rubus fruticosus* leaves obtained through HPLC analysis. Data are expressed as mg/l and are the means of three independent analyses.

Phenolic compounds	***J. oxycedrus***	***R. fruticosus***
Gallic acid	1.38 ± 0.01	0.13 ± 0.01
Caffeic acid	n.d.[a]	2.04 ± 0.04
Protocatechuic acid	0.32 ± 0.02	n.d
p-Hydroxybenzoic acid	0.81 ± 0.01	n.d
Vanillic acid	10.51 ± 0.16	n.d
Chlorogenic acid	n.d	6.22 ± 0.07
p-Coumaric acid	n.d	0.63 ± 0.01
(−)-Epicatechin	0.51 ± 0.01	0.11 ± 0.01
(+)-Catechin	4.86 ± 0.01	n.d
Rutin	6.95 ± 0.01	29.88 ± 0.39
Astringin	n.d	2.41 ± 0.02
Apigenin	7.66 ± 0.04	n.d
(−)-Epigallocatechin gallate	0.72 ± 0.16	n.d
Total	**33.72**	**41.42**

[a]: not detected

The dominant phenolic acid in *J. oxycedrus* needles extract was vanillic acid, with a concentration of 10.51 mg/l. Among flavonoids, the most abundant were apigenin and rutin, whose amounts were 7.66 and 6.95 mg/l, respectively. Regarding *R. fruticosus* leaves PE, the dominant phenolic acid was chlorogenic acid (6.22 mg/l), while rutin was the most abundant flavonoid, with a concentration of 29.88 mg/l. Except for caffeic acid and astringin, the amount of all other identified compounds was lower than 1 mg/l in this PE.

Previously, it was reported that *J. oxycedrus* berry extracts have smaller content of total phenolics than the counterparts of the same genera (Živić et al., 2019). Some authors recorded that the extraction by polar solvents, such as ethanol (used also in this extraction), increased the total phenolic yield during extraction. A similar output was reported by Orhan et al. (2011) for *J. oxycedrus* leaves ethanol extracts. Moreover, the authors found that ethanolic extract of *J. oxycedrus* leaves had the highest amount of total phenols (206.19 ± 9.04 mg/g) among the five juniper species tested.

However, regarding the chemical composition of juniper extract there are a few reports. Mrid et al. (2019) identified salicylic acid (> 30 mg/g of dry weight of sample) and rutin (10.8 mg/g) as the most abundant compounds in *J. oxycedrus* needles methanolic extracts. Besides, the authors reported a high level of hesperidin (2.8 mg/g) and low concentrations of caffeic, p-coumaric and p-hydroxybenzoic acids (< 2 mg/g). Dziedzinski et al. (2020) found that *J. communis* shoots

were particularly rich in caffeic, ferulic, chlorogenic and gallic acids (> 1000 μg/g), while showed a small abundance of flavonoids (< 1 μg/g).

The blackberry fruits were intensively studied and were found to be a rich source of biologically active phenolic compounds, particularly gallic acid and rutin (Ferlemi and Lamari, 2016; Radovanović et al., 2013). On the other hand, a small number of publications indicated that the blackberry leaves could also be a good source of various groups of phenolic compounds (Ferlemi and Lamari, 2016; Oszmiański et al., 2015). In general, blackberry leaves are rich in p-hydroxybenzoic and hydroxycinnamic acids (caffeic, gallic, ferulic, gentisic, vanillic, p-coumaric and others), ellagitannins and flavonoids, such as quercetin, mirycetin, luteolin, apigenin, kaempferol, catechin, and epicahechin. Oszmiański et al. (2015) identified and quantified flavonoid derivatives of quercetin, kaempferol, luteolin and apigenin in different *Rubus* species, ranged from from 8.68 to 61.27 mg/g of dry matter. They offered an overview of phenolic composition of 26 different wild blackberry leaves, although *R. fruticosus* was not included, and identified 33 phenolic compounds, including 15 flavonoids (5 kaempferol and 10 quercetin derivatives), 9 phenolic acid derivatives, 3 derivatives of ellagic acid and 2 flavones (apigenin and luteolin). Gudej and Tomczyk (2004) reported the content of quercetin and kaempferol in *R. fruticosus* leaves, that was found in ranges respectively of 0.16-0.31 and 0.11-0.15% of dry weight of samples.

In Table 5.2 the characterisation of the EO obtained from *J. oxycedrus* needles is reported. Only molecules that represented at least 0.5% of the total area (34 out of 119 identified compounds) are listed.

The majority of identified molecules were terpenes or terpenoids, representing 88.11% of the total peak area. Limonene, α-pinene and manoyl oxide accounted for more than 30% of peak areas (13.59, 10.71 and 8.41%, respectively), while 3-carene, 4(15),5-muuroladiene and α-curcumene represented 4.12, 3.24 and 3.50% of EO components. Minor amounts of δ-cadinene, β-pinene and β-myrcene were also detected.

The composition of *Juniperus* spp. EO reported in the literature is highly variable and depends on the species, the geographic area and the part of the plant used for the production. α-pinene was the major compound in hydrodistilled *J. communis* berries EO from Portugal, followed by β-pinene and limonene (Falcão et al., 2018). The same authors also tested two commercial samples of the same EO in which α-pinene and β-pinene were again among the most important constituents, but their concentrations were markedly lower. Medini et al. (2013) studied the EO obtained from needles of *J. oxycedrus* harvested in different Tunisian localities and found that the major constituents were α-pinene (which ranged from 16.0 to 49.6%), sabinene (0-12.1%), p-cimene (0-14.5%), germacrene D (0.5-9.0%) and manoyl oxide (2.5-6.4%). α-pinene was again the

main terpene found in Italian *J. oxycedrus* needles EO, ranging from 31.5 to 61.8%, followed by germacrene D (Najar et al., 2020). Finally, a Bulgarian EO from *J. oxycedrus* leaves presented a composition similar to the EO characterised in this thesis and, in addition to α-pinene and limonene, relevant amounts of manoyl oxide, caryophyllene oxide, abietatriene, curcumene and β-caryophyllene were detected (Semerdjieva et al., 2020).

Table 5.2: Composition of essential oils (EO) of *Juniperus oxycedrus* needles obtained through GC-MS analysis. Data are expressed as relative percentages (± standard deviation) of each peak area with respect to the total peak area and are the means of three independent analyses. Only peak with area higher than 0.5% are reported.

No	RT[a]	Compounds	%
1	18.33	3-carene	4.12 ± 0.09
2	19.12	α-pinene	10.79 ± 0.19
3	21.38	β-pinene	0.88 ± 0.02
4	22.08	β-myrcene	0.94 ± 0.04
5	23.95	*o*-cymene	0.61 ± 0.03
6	24.38	Limonene	13.59 ± 0.09
7	27.41	*p*-menth-4(8)-ene	1.14 ± 0.01
8	27.89	Linalool	0.56 ± 0.01
9	29.37	Campholenic aldehyde	1.18 ± 0.05
10	30.12	(*E*)-pinocarveol	1.00 ± 0.01
11	30.42	Verbenol	1.68 ± 0.05
12	30.58	1.3-cycloheptadiene	0.55 ± 0.01
13	31.47	1.3.5-heptatriene	1.29 ± 0.03
14	32.15	*p*-cymen-8-ol	1.06 ± 0.05
15	32.69	*p*-menth-1-en-8-ol	0.65 ± 0.01
16	33.00	α-thujenal	0.73 ± 0.06
17	33.25	Bornyl acetate	1.21 ± 0.01
18	33.67	(−)-verbenone	1.11 ± 0.06
19	34.06	(*Z*)-carveol	1.68 ± 0.03
20	35.27	(+)-carvone	1.03 ± 0.01
21	42.84	α-cedrene	1.94 ± 0.05
22	43.57	Caryophyllene	0.60 ± 0.01
23	44.01	4(15),5-muuroladiene	3.24 ± 0.04
24	45.02	α-caryophyllene	0.53 ± 0.01
25	46.00	α-curcumene	3.50 ± 0.02
26	47.48	α-amorphene	2.03 ± 0.07
27	47.79	δ-cadinene	2.04 ± 0.03
28	48.53	α-copaene-11-ol	0.55 ± 0.01
29	48.63	α-calacorene	1.04 ± 0.06
30	50.31	Caryophyllene oxide	1.38 ± 0.01
31	51.00	Cedrol	0.84 ± 0.05
32	54.02	Farnesol	0.75 ± 0.01
33	60.15	Manoyl oxide	8.41 ± 0.06
34	61.30	ar-abietatriene	1.19 ± 0.01
		Total identified compounds	**88.11**

[a]: retention time (min)

The EO obtained from *R. fruticosus* leaves was characterised by the identification of 118 molecules (representing 90.94% of the total peak area). In Table 5.3 are reported the 36 compounds that accounted for a percentage higher than 0.5%.

The major components of this EO were monoterpenes, such as geraniol (13.67%), β-citronellol (4.61%), linalool (4.13%), α-terpineol (3.05%) and citral (2.40%). Phytol, an acyclic diterpene alcohol, was present at a concentration of 4.87%, while β-ionone, typically giving a scent of violet and involved in vitamin A metabolism, accounted for 3.68% and olivetol for 3.02%. Several alkanes were also detected (tetradecane, hexadecane, eicosane and heneicosane), together with fatty acids (octanoic, decanoic, dodecanoic, tetradecanoic and hexadecenoic acid). Interestingly, also methyl salicylic acid was detected at a concentration of 1.25%.

The literature concerning the EO composition of *Rubus* spp. is limited. In their study, Wajs-Bonikowska et al. (2017) compared *R. fruticosus* pomace EO obtained from hydrodistillation or using supercritical CO_2. The main constituents were non-saturated aliphatic aldehydes. Among terpenes, α-copaene and p-cymene were the most relevant, but their concentration was rather low. The composition of *R. pungens* leaves EO was characterised by the presence of 36% of sesquiterpenes, including γ-elemene and β-caryophyllene (Zhang et al., 2017b). Another study demonstrated that the vegetative stage of the plant dramatically influenced the EO composition of *R. ulmifolius*. Anyway, the most relevant compounds detected were α-pinene, 1,8-cineole, linalool, geraniol and, among aldehydes, (E)-2-hexenal and nonanal (Caponi et al., 2002).

Table 5.3: Composition of essential oils (EO) of *Rubus fruticosus* leaves obtained through GC-MS analysis. Data are expressed as relative percentages (± standard deviation) of each peak area with respect to the total peak area and are the means of three independent analyses. Only peak with area higher than 0.5% are reported.

No	RT[a]	Compounds	%
1	27.92	Linalool	4.13 ± 0.33
2	31.49	1-nonanol	0.59 ± 0.03
3	31.84	Octanoic acid	0.57 ± 0.32
4	32.69	α-terpineol	3.05 ± 0.29
5	32.83	Methyl salicylate	1.25 ± 0.02
6	33.22	Decanal	1.04 ± 0.12
7	34.36	β-citronellol	4.61 ± 0.15
8	35.01	Citral	2.40 ± 0.22
9	35.70	Geraniol	13.67 ± 1.09
10	37.82	Geranyl formate	0.50 ± 0.12
11	38.05	Undecanal	0.58 ± 0.09
12	40.75	n-decanoic acid	1.04 ± 0.50
13	41.76	(E)-β-damascenone	0.82 ± 0.13
14	42.20	Tetradecane	0.59 ± 0.13
15	44.49	Geranylacetone	0.63 ± 0.11
16	46.14	β-ionone	3.68 ± 0.55
17	46.88	α-farnesene	2.01 ± 0.24
18	47.41	3-amino-2-cyclohexen-1-one	0.95 ± 0.04
19	47.70	Olivetol	3.02 ± 0.24
20	49.03	Dodecanoic acid	3.11 ± 1.66
21	49.10	Nerolidol	1.03 ± 0.59
22	50.32	Hexadecane	0.62 ± 0.09
23	51.03	Linalyl acetate	0.77 ± 0.11
24	53.76	Hexadecanal	3.27 ± 0.40
25	54.86	Tetradecanoic acid	0.81 ± 0.45
26	56.12	Tetradecanal	2.18 ± 0.25
27	56.70	Hexahydrofarnesyl acetone	1.33 ± 0.19
28	57.71	Eicosane	0.80 ± 0.14
29	58.11	16-octadecenal	1.87 ± 0.28
30	58.17	Farnesyl acetone	0.65 ± 0.05
31	58.88	n-hexadecanoic acid	1.17 ± 1.00
32	61.23	5-Octadecene	0.57 ± 0.03
33	61.47	Heneicosane	0.89 ± 0.25
34	61.90	Phytol	4.87 ± 0.79
35	62.49	Oleic acid	2.02 ± 1.02
36	64.45	Octadecanale	0.52 ± 0.03
		Total identified compounds	**90.49**

[a]: retention time (min)

5.1.3.2 Effect of plant derivatives on *Listeria monocytogenes* growth kinetics

Firstly, PE and the EO of *R. fruticosus* and *J. oxycedrus* were tested to define their MIC against *List. monocytogenes* (Table 5.4). The results indicated that MIC was 1.5 mg/ml for the EO of *R. fruticosus* and 2 mg/ml for the EO of *J. oxycedrus* and for both PE.

Table 5.4: MIC determination to describe the antimicrobial activity of tested plant derivatives (PE and EO obtained from *J. oxycedrus* and *R. fruticosus*) against *List. monocytogenes* Scott A.

	***J. oxycedrus* MIC (mg/ml)**		***R. fruticosus* MIC (mg/ml)**	
Target microorganism	**PE**	**EO**	**PE**	**EO**
List. monocytogenes Scott A	2	2	2	1.5

As already observed, the comparison between MIC of plant derivatives from different studies can be difficult because of the variable composition of derivatives and especially the lack of standardised test procedures (Balouiri et al., 2016). In addition, literature about the MIC of the two species considered is limited. In any case, a MIC of 2 mg/ml against *List. monocytogenes* has been reported for gallic acid (Skroza et al., 20190). Cosentino et al. (2003) found a minimum lethal concentration of *J. oxycedrus* EO at 900 mg/l for *List. monocytogenes* (and 250 mg/l for δ-carene), while lower MIC (approx. 32 mg/l) were observed by Najar et al. (2020), but in this latter case the time of incubation was 24 h, against the 48 h of these analyses.

To better understand the effects of these plant derivatives on *List. monocytogenes*, the target microorganism was inoculated in BHI medium added with a sub-lethal dose (corresponding to 50% of MIC) of these PE and EO and the samples were incubated at 20°C. The choice to use sub-lethal concentrations was aimed to assess the ability of cells to repair the damages induced by plant derivatives and the effects of the presence of these substance on the growth dynamics. The culturability was monitored over time by plate counting and compared with the control grown in the absence of inhibiting products.

In Figure 5.1 experimental points and the relative fitted models for each condition are reported.

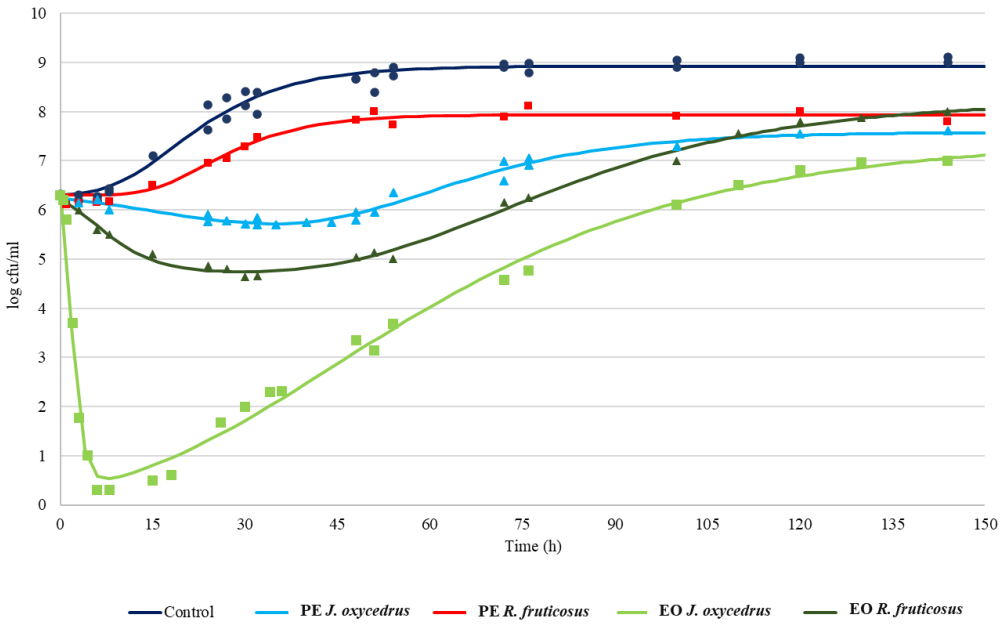


Figure 5.1: *List. monocytogenes* Scott A growth kinetics during incubation at 20°C in the presence of different concentrations of plant derivatives: 1 mg/ml of PE of *J. oxycedrus* needles or *R. fruticosus* leaves, 1 mg/ml of EO of *J. oxycedrus* needles and 0.75 mg/l of *R. fruticosus* leaves EO. The points represent the experimental data obtained by plate counting, while curves are the relative fitted models obtained with Gompertz equation.

The distribution of the observations followed two distinct trends. The first, that characterised the control and the sample with *R. fruticosus* leaves PE, represented a classical growth curve, with three distinct phases: lag, exponential and stationary phase. In this case, the experimental data were fitted with the classical Gompertz equation (Zwietering et al., 1990). Conversely, the remaining samples showed, to a different extent, a first step in which cells decreased their culturability, after which the growth restarted with an exponential phase reaching the stationary phase and the maximum cell concentration. These experimental data were therefore fitted a double-peaked Gompertz model, in which the first step describes the diminution of cell counting and the second the increase of cell number up to the reaching of the stationary phase (Tattershall et al., 2021). These estimated parameters are reported in Table 5.5, together with the maximum cell concentration attained according to the models and some diagnostics of fitting.

Table 5.5: *List. monocytogenes* Scott A growth parameters in the presence of different plant derivatives (PE or EO), estimated by modelling the data obtained from plate counting (log cfu/ml) with the Gompertz equation. The maximum cell concentration attained according to the model and some diagnostics of fitting are also reported.

Samples	k	$A1^a$	$\mu_{max}1^a$	$\lambda1^a$	$A2$	$\mu_{max}2$	$\lambda2$	Max cell load	R	RMSE
Control	6.30	–	–	–	2.62	0.093	7.84	8.92	0.994	0.005
J. oxycedrus PE	6.30	− 0.79	− 0.020	− 1.11	2.07	0.043	40.66	7.58	0.982	0.007
R. fruticosus PE	6.30	–	–	–	1.64	0.066	14.28	7.94	0.988	0.007
J. oxycedrus EO	6.30	− 1.69	− 0.078	− 2.83	3.63	0.050	43.67	7.99	0.994	0.015
R. fruticosus EO	6.30	− 6.20	− 1.926	0.38	7.33	0.079	10.03	7.33	0.995	0.046

[a]: parameters estimated only for the double-peaked Gompertz model used when the initial cell concentration decreased before the growth started again

The presence of *R. fruticosus* leaves PE determined a prolongation in the lag phase with respect to the control (14.28 h *vs*. 7.84 h), a slower exponential phase growth rate (0.066 (log cfu/ml)/h *vs*. 0.093 (log cfu/ml)/h) and a final concentration of 1 log unit lower than the control (7.94 log cfu/ml *vs*. 8.92 log cfu/ml). The PE of *J. oxycedrus* needles was more effective, and determined an initial decrease of the population culturability, even if low (0.79 log cfu/ml). Then, after 40.66 h, the cell number started to increase, although with a maximum growth rate of 0.043 (log cfu/ ml)/h, lower with respect to the control and the sample containing *R. fruticosus* PE, and reached a maximum cell concentration of 7.58 log cfu/ml.

The antimicrobial activity of PE from *Rubus* spp. was already described. In particular, the anti-listerial effect of ethanolic extract from *R. discolor* was demonstrated *in vitro* and in yoghurt by Veličković et al. (2021), while the blackberry juice from *R. fruticosus* showed an inhibiting effect on *List. monocytogenes*, *S.* Typhimurium and *E. coli* O157:H7 in milk and in BHI medium (Yang et al., 2014). The antimicrobial potential of molecules, including also those found in the extracts studied in this thesis (such as gallic acid, vanillic acid, protocatecuic acid, rutin, apigenin and caffeic acid) has been recently reviewed by Oulahal and Degraeve (2022). For example, a MIC value of 2 mg/ml of gallic acid against *List. monocytogenes* was reported (Skroza et al., 2019). The role of phenolic compounds, used as vegetable extracts or pure molecules after isolation, in inhibiting this pathogen in model systems and foods was also described by Zamuz et al. (2021). The authors stated that, among the wide array of these compounds, some were able to exert protective effects against *List. monocytogenes*, in particular thanks to the presence of stilbenes (resveratrol), cinnamic acids (cinnamul-3,4-dihydroxy-α-cyanocinnamate and caffeic acid 1,1-dimethylallyl ester), benzoic acids (butyl gallate and 3,4-dihydroxy-benzoic acid methyl ester) and flavonoids (epigallocatechin gallate). Concerning the mechanism of action, it has been recently demonstrated that phenolic extract from *Taraxacum officinale* containing rutin, caffeic acid and chlorogenic acid caused in *Staph. aureus* membrane depolarisation and permeabilisation and altering the intracellular enzymatic activities (Xu et al., 2021). Quercetin and chlorogenic acid resulted effective against *List.*

monocytogenes by involving redox imbalance which determined mortality increase (Jobim et al., 2014). Cushnie and Lamb reported that, at cytoplasmic level, apigenin could alter the activity of DNA gyrase and rutin interferes with topoisomerases, while catechins mainly perturb the membrane functionality.

Both EO were more effective in inhibiting the growth of *List. monocytogenes*. In particular, the EO from *R. fruticosus* leaves determined a loss of culturabilty of almost the whole population (cell load reduction of about 6 log cfu/ml) in a few hours of incubation. However, after about 10 h multiplication of survivors began and determined a maximum estimated cell concentration of 7.33 log cfu/ml. The EO of *J. oxycedrus* needles showed a lower impact on initial cell culturability. Nevertheless, it determined a reduction of the initial cell concentration of 1.69 log cfu/ml, after which cell number increased reaching a final value of 7.99 log cfu/ml.

This plant derivative contained several terpenes, many of which have a known antimicrobial effect. β-myrcene, linalool, citral, geraniol, 3-carene and cymene showed antimicrobial activity against several microorganisms (Masyita et al., 2022). A *J. communis* EO rich in α-pinene (47.8%) used in a marinade was effective in reducing *List. monocytogenes* concentration in beef meat (Vasilijević et al., 2019), while another EO of the same species (containing 14.1% β-myrcene, 9.5% sabinene, 8.4% limonene and 5.4% α-amorphene) was able to avoid the proliferation of this pathogen in fermented sausages (Tomović et al., 2020). Semerdjieva et al. (2020) showed that the *J. oxycedrus* leaves EO was characterised by a good antimicrobial activity against *Staph. aureus*. The same inhibiting effect against *Staph. aureus* was observed also in the EO obtained from Tunisian *J. oxycedrus* leaves, while the action was scarce against the Gram-negative *E. coli* and *S. enterica* serovar Enteritidis (Medini et al., 2013).

As observed in Table 5.2, terpenes, and in particular limonene, α-pinene, manoyl oxide and 3-carene, were the most important constituents of the *J. oxycedrus* EO (approx. 45% of the total). α-pinene and 3-carene were also the main constituents of a *Cupressus sempervirens* EO, which inhibited *Staph. aureus* by affecting the activity of the efflux pump (Akermi et al., 2022). In addition, several papers report the possible antimicrobial action of molecules present in *J. oxycedrus* EO (da Silva et al., 2021; Masyita et al., 2022). On the other hand, independently of the efficacy of the various oils reported in the literature, it is well known that the inhibitory or bactericidal activity depends on their composition and the interaction between the components, which can bring to relevant synergistic effects (Yousefi et al., 2020). In fact, components with limited antimicrobial activity if considered alone, could show a relevant inhibition if used in combination, as observed for linalool and β-pinene on *Saccharomyces cerevisiae* (Tabanelli et al., 2014).

The composition of *R. fruticosus* EO was characterised by the presence of terpenoids, such as linalool, α-terpineol, β-citronellol and geraniol, whose antimicrobial activity was demonstrated (de Lira et al., 2020; He et al., 2022; Nogueira et al., 2021). Moreover, among other constituents, the antimicrobial activity of phytol and short chain fatty acids (such as octanoic, decanoic, dodecanoic acid) were also described (Churchward et al., 2018).

5.1.3.3 Effect of plant derivatives on *Listeria monocytogenes* viability

With the aim to better investigate the impact of plant derivatives on *List. monocytogenes* physiological state, cells were analysed with a flow cytometric protocol to highlight the occurrence of different subpopulations characterised by different physiological states. In Figure 5.2 the results after 0, 24, 48 and 72 h are represented. In particular, the data of cell culturability (expressed as log cfu/ml predicted by the models) were compared to the total cells detected by flow cytometry. For this latter, also the relative percentages of viable, injured and dead cells for each condition are reported.

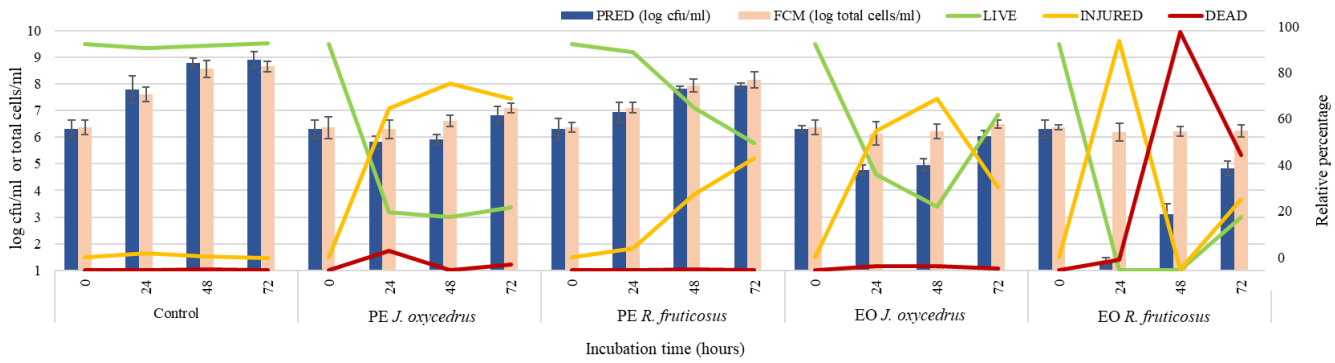


Figure 5.2: *List. monocytogenes* Scott A growth in the presence of different plant derivatives of *J. oxycedrus* and *R. fruticosus* (PE or EO) after 24, 48 and 72 h of incubation at 20°C. The histograms represent the comparison between the data of cell culturability (expressed as log cfu/ml predicted by the models) and the total cells detected by flow cytometry (total cells/ml). For these latter, also the relative percentages of viable, injured and dead cells for each condition (as green, yellow and red lines, respectively) are reported.

Regarding the control, the data of sampling and flow cytometric analysis were similar, without significant differences and, in all cases, the viable cells represented about 94% of the total population. In the sample added with the *J. oxycedrus* needles PE, the data of viability were coherent with the culturability predicted. This extract after 24 h mainly determined the presence of a high percentage of injured cells (67.65%), while live cells represented 24.26% of the total events. Interestingly, this ratio between alive and injured cells remained quite constant throughout the incubation time (72 h). The presence of this injured population can be responsible for the extended lag phase duration (approx. 40 h *vs*. 8 h for the control), due to the need to overcome cell damage

before starting multiplication. Indeed, similar behaviors were previously observed for List. monocytogenes after exposure to environmental stresses (Sibanda and Buys, 2017).

The PE of *R. fruticosus* leaves, which caused a reduction of 1 log unit of the final maximum cell concentration, determined an increasing proportion of injured cells (from 9.09% after 24 h to 46.83% after 72 h). The different trend of cell injury between the two PE was likely due to their different composition. In fact, in this case the occurrence of injury in the cell population did not result in a remarkable prolongation of lag phase (about 14 h). However, the increasing ratio of injured cells overtime could be explained by the effect of *R. fruticosus* PE constituents, particularly rutin and chlorogenic acid, whose potential to affect cell membrane permeability (thus allowing higher retention of propidium iodide, used in this analysis to detect injured cells) was demonstrated for some Gram-positive bacteria (Xu et al., 2021).

Concerning *J. oxycedrus* needles EO, data of culturability after 24 and 48 h of incubation were lower if compared with the total cells detected by flow cytometry. This could be due to a decrease of cells recognised as live and the relative increase in the percentage of injured cells (58.24 and 71.59% after 24 and 48 h, respectively), being the latter likely responsible for the significant increase of lag phase (about 44 h), as previously observed for the *J. oxycedrus* needle PE. In addition, the number of total cells did not markedly change, indicating that the increase of culturable cells within the first 72 h of incubation depended on the recovery of injured cells (which passed from 71.59% at 48 h to 34.51% at 72 h) rather than on a multiplication. After 72 h an opposite trend was observed, suggesting an active response of the target strain to overcome the stress induced by the presence of these tested plant derivatives and to start to multiply, as stated by the growth kinetics (Figure 5.1).

The EO of *R. fruticosus* leaves showed the more evident effects. Besides, in this case, the number of total cells detected through flow cytometry did not significantly change during 72 h incubation. The predicted culturability at 24 h was 1.31 log cfu/ml, while the viability was higher, *i.e.* 2.88 log cell/ml, which corresponded to 0.05% of cells recognised as live in the total population. Under this condition, the majority of cells was injured (95.65%). More than 99% of cells were classified as dead and 0.1% as alive at 48 h. After 72 h the data of culturability increased, and also the ratio of live cells, that reached 20.02% of the total population. Based on these results, it was possible hypothesise that *R. fruticosus* EO was the only plant derivative able to exert a bactericidal effect against *List. monocytogenes*, *i.e.* to induce a relevant increase of its dead cells. Indeed, the initial discrepancy between viability and culturability could suggest the occurrence of viable but not culturable cells (VBNC), a great safety concern because of the ability to reverse to a viable status (Montanari et al., 2021b).

However, considering both plate counting and flow cytometry analysis, it seems that the increase of culturability (log cfu/ml) and viability (% of live cells) observed starting from 48 h of incubation was likely due to the multiplication of the few cells survived to the effect of this EO, rather than a resuscitation phenomenon.

Conversely, the other plant derivatives tested were able to sub-lethally damage *List. monocytogenes* cells, determining different extents of cell injury that indicate differences in the mechanism of antimicrobial action, particularly in terms of cell membrane integrity. This confirmed the suitability of flow cytometry and fluorescent staining procedures in the study of cell injury caused by stress factors to better elucidate the different patterns of metabolic responses and the potential safety risks (Sibanda and Buys, 2017).

5.1.4 Conclusions

The plant derivatives from *J. oxycedrus* needles and *R. fruticosus* leaves considered in this study were able to slow down the growth kinetics of *List. monocytogenes*, monitored by plate counting, even if added at concentrations corresponding to half of MIC. In general, both PE and EO significantly reduced the final maximum cell culturability of approx. 1 log unit. PE were less effective than EO in limiting the growth performance of the target microorganism. Indeed, no loss of culturability was observed for the PE of *R. fruticosus* leaves and only a weak decline was observed for *J. oxycedrus* needles PE. The anti-listerial activity was more relevant using EO, in aprticular the one derived from *R. fruticosus* leaves. This latter caused the most severe effects, since it was able, in a few hours of incubation, to almost completely inhibit the cell culturability. According to the flow cytometry analysis, the presence of this EO was able to induce the death of the major part of cells (> 99%). Concerning the other plant derivatives, the discrepancy observed in some cases between viability and culturability could indicate the presence of cells not able to grow in culture media (at least in the adopted conditions), whose fate needs to be further investigated for a deeper comprehension of their possibility to revert to culturable status, contributing to an overestimation of the effect of these antimicrobial substances.

The obtained results increase the knowledge of these underused raw materials such as blackberry leaves and juniper needles, that can be exploited in food production and other industrial sectors.

Data published as:

Barbieri, F., Montanari, C.*, Šimat, V., Skroza D., Čagalj, M., Smole-Možina, S., Bassi, D., Gardini, F., Tabanelli, G. (2022). Effects of *Rubus fruticosus* and *Juniperus oxycedrus* derivatives on the growth of *Listeria monocytogenes*: comparison between culturability and viability. *Scientific Reports*, 12, 13158. doi: 10.1038/s41598-022-17408-4.

5.2 Survival, growth and biogenic amine production of *Enterococcus faecium* FC12 in response to extracts and essential oils of *Rubus fruticosus* and *Juniperus oxycedrus*

5.2.1 Introduction

Enterococci are LAB that can be found in different habitats and they are highly competitive in harsh conditions due to their relevant salt, pH and broad temperature range tolerance. For this reason, they are part of the microbiota of several foods of animal origin, such as cheeses and sausages (Franz et al., 2011; Graham et al., 2020). However, their presence is controversial (Almeida-Santos et al., 2021). In fact, some enterococci have been described for their probiotic features and capability of producing active bacteriocins against pathogens (Graham et al., 2020; Nami et al., 2019). On the other hand, this genus is known for its antibiotic-resistance, which can be transferred to other microorganisms, the potential presence of virulence factors, such as cytolysins, aggregation substances, and gelatinase extracellular surface proteins (Hollenbeck and Rice, 2012; Rossi et al., 2014).

In addition, enterococci produce biogenic amines (BA), some basic nitrogenous molecules derived from the decarboxylation of amino acids (Anderegg et al., 2020; Barbieri et al., 2019; Gardini et al., 2016). Although these molecules can be formed in controlled amounts by the human body, where they play regulatory roles in different physiological activities, their excessive intake through diet can cause severe symptoms depending on the health status of the consumer (EFSA, 2011).

Among BA, histamine and tyramine are those with the most severe acute effects and they are responsible for the “fish poisoning” symptoms (Hungerford, 2021) and the “cheese reaction” syndrome (Wójcik et al., 2021), respectively. Tyramine is the result of the decarboxylation of tyrosine and may be found in relevant amounts in foods, such as cheeses and fermented sausages (Andersen et al., 2019). The tyraminogenic potential is considered a species characteristic in *Ent. faecalis* (Barbieri et al., 2019; Bargossi et al., 2017; EFSA, 2011), but it is also extremely widespread among *Ent. faecium*, *Ent. mundtii* and *Enterococcus durans* (Gatto et al., 2016; Ladero et al., 2012). Therefore, strategies that can control the growth of these bacteria are a crucial point for many fermented products.

In recent years, the antimicrobial activity of plant extracts has been deeply studied, following the consumer demand for “green” preservatives. It is well known that many plant extracts can contain molecules with a relevant antimicrobial activity, which depends, in the first instance, on their composition. Many variables, including seasonal, geographical and agronomic factors, as well

as the mode of extraction and part of the plant, influence the qualitative and quantitative presence of antimicrobial compounds and, in turn, their effectiveness (Gottardi et al., 2016; Ni et al., 2021).

The Mediterranean area is characterised by high plant biodiversity and many species are known to produce antimicrobial substances. *R. fruticosus*, belonging to the family *Rosaceae*, is extremely diffused and popular for blackberry production. Several parts of the plant, such as fruits, leaves and young shoots, have been widely applied in traditional medicine (Verma et al., 2014). In addition, their PE showed a relevant antioxidant activity, due to the presence of phenolic acids, flavonoids (anthocyanins, flavonols and tannins), carotenoid and organic acids (Krzepiłko et al., 2021). Many of these antioxidant molecules can also exert antimicrobial activity. Aqueous and acetone extracts of *R. discolor* were effective in inhibiting the growth of many microorganisms, including *List. monocytogenes* (Veličković et al., 2021), while the methanolic extract of stem, leaf, fruits, and roots of *R. fruticosus* was active against *E. coli*, *S.* Typhi and *Staph. aureus*, with none or scarce effects against yeasts and moulds (Riaz et al., 2011).

Junipers are another group of plants typical of the Mediterranean maquis. Extracts of *J. communis*, *J. turbinata*, *J. deltoides* and *J. oxycedrus* were extensively studied for their antioxidant activity and antimicrobial potential (Boudiba et al., 2021; Dziedzinski et al, 2020). Studies were mainly focused on EO extracted from leaves and berries, in which α-pinene is usually the major constituent, followed by myrcene, sabinene, limonene, germacrene D, δ-cadinene and other terpenes and terpenoids (Falcão et al., 2018). Prickly juniper, *J. oxycedrus*, is a typical Mediterranean plant, whose EO was effective in inhibiting or reducing the growth of some Gram-positive bacteria. Aqueous extract of *J. oxycedrus* needles did not show antimicrobial effect, while its methanol extract inhibited, to different extent, the growth of 57 strains belonging to 24 species, including *Staph. aureus*, *Enterobacter* spp. and *Bacillus* spp. (Medini et al., 2013; Semerdjieva et al., 2020).

Given these considerations, in this part of the thesis, analyses were performed in order to evaluate effects of the same PE and EO plant derivatives previously tested against *List. monocytogenes*. In this case these bio-active compounds were evaluated against the strain *Ent. faecium* FC12, a strong tyramine producer (Bargossi et al., 2015a,b). The growth dynamics in the presence of a sub-lethal concentration of the plant derivatives were modelled to highlight their antimicrobial effects. The accumulation of tyramine was also monitored during the incubation. Eventually, *Ent. faecium* cells grown in these conditions were analysed through flow cytometry to investigate the effect of these plant derivatives on cell viability and culturability.

5.2.2 Materials and methods

5.2.2.1 *Enterococcus faecium* FC12

The strain *Ent. faecium* FC12 used to perform these analyses was isolated from a traditional Italian cheese and belonged to the Department of Agricultural and Food Sciences (University of Bologna). Moreover, it was previously described as a high tyramine producer (Bargossi et al., 2015a,b). This strain was maintained in BHI medium (Oxoid) added with 20% (w/v) glycerol at -20°C until the analyses. Before each experiment, the strain was pre-cultivated twice in BHI medium for 24 h at 30°C.

5.2.2.2 Plant derivatives and minimum inhibiting concentration (MIC) determination

The PE and EO used in these analyses were obtained *R. fruticosus* leaves and *J. oxycedrus* needles and their chemical composition was previously described and characterised (Paragraph 5.1.3.1). Firstly, the *in vitro* antimicrobial activity of these plant derivatives against the target strain was assessed to determine the MIC by broth microdilution method using microtiter plates according to the protocol described in the Paragraph 5.1.2.5. The data were collected after 48 h of incubation at 30°C and the MIC was defined as the lowest concentration of plant derivatives was able to prevent a visible microbial growth in the well.

5.2.2.3 Growth kinetics of *Ent. faecium* FC12 in the presence of tested plant derivatives

The target microorganism was inoculated in BHI broth with a final concentration about 6 log cfu/ml and sub-lethal concentrations of plant derivates (50% of MIC) were added to evaluate their effect on the growth kinetics. In particular, 1 mg/ml of extract was used in each condition, except for *R. fruticosus* EO, whose concentration was 0.75 mg/ml. Samples were incubated at 20°C and their culturability was assessed by sampling at specific time for 96 h. For sampling (Paragraph 3.1.2.3), appropriate decimal dilutions were plated onto BHI agar medium and incubated at 30°C for 48 h. The analyses were performed in triplicate and the data obtained from plate counting were modelled with the classical or double-peaked Gompertz equation (Tattershall et al., 2021; Zwietering et al., 1990). Parameters were described in detail in Paragraph 5.1.2.6. Data modelling was performed using Statistica 8.0 software (Statsoft Inc.).

5.2.2.4 Evaluation of biogenic amines production during samples incubation

The formation of tyramine and 2-phenylethylamine in the different tested conditions was monitored collecting samples at defined time (0, 24, 48, 72 and 96 h). The cell-free supernatants, after a derivatisation reaction, were analysed using a HPLC Agilent Instrument 1260 Infinity with

the automatic injector (G1329B ALS 1260, loop of 20 µl), equipped with a UV detector (G1314F VWD 1260) set at 254 nm, following the method described in Paragraph 3.2.2.4.

BA concentrations were expressed as mg/l with reference to a respective standard calibration curve. All the samples were analysed in triplicate.

5.2.2.5 Flow cytometry analysis

To test cell viability, the samples were collected after 24, 48, and 72 h of incubation and analysed with a flow cytometer Accuri C6 (BD Biosciences), following the protocol reported by Arioli et al. (2019) and described in Paragraph 5.1.2.7. The data obtained were analysed using the BD ACCURITM C6 software version 1.0 (BD Biosciences).

5.2.3 Results and discussion

5.2.3.1 Evaluation of plant derivatives effects against *Ent. faecium* FC12

The same PE and EO obtained from *R. fruticosus* and *J. oxycedrus* and previously characterised were employed to define the MIC of the plant derivatives against *Ent. faecium* FC12, that was 1.5 mg/ml for *R. fruticosus* EO and 2 mg/ml for *J. oxycedrus* EO and both PE (Table 5.6).

Table 5.6: MIC determination to describe the antimicrobial activity of tested plant derivatives (PE and EO obtained from *J. oxycedrus* and *R. fruticosus*) against *Ent. faecium* FC12.

	***J. oxycedrus* MIC (mg/ml)**		***R. fruticosus* MIC (mg/ml)**	
Target microorganism	**PE**	**EO**	**PE**	**EO**
Ent. faecium FC12	2	2	2	1.5

After MIC determination, the target microorganism was inoculated into BHI medium and incubated at 20°C. Samples were added with sub-lethal concentrations of plant derivatives (50% of MIC) in order to activate a metabolic response due to the stress conditions. The growth dynamics were monitored by sampling and compared with the control grown in the absence of plant derivatives. In Figure 5.3 the experimental points and the corresponding fitted models are reported.

Data showed two different kinetics trends. In particular, the control and the samples containing PE presented a typical growth curve, characterised by three distinct steps: lag, exponential and stationary phase. In contrast, the samples containing EO showed an initial part in which cells decreased their culturability. After that, cell concentration increased reaching an exponential phase and a subsequent stationary phase. In the first case, the experimental data were fitted with the classical Gompertz equation (Zwietering et al., 1990), while in the second case, a double-peaked Gompertz equation was chosen (Tattershall et al., 2021).

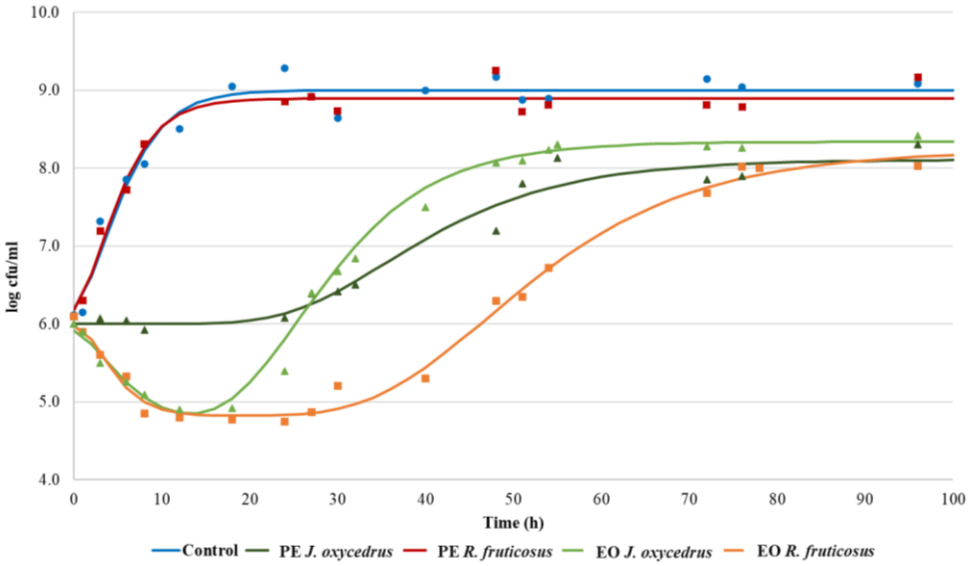


Figure 5.3: Growth kinetics of *Ent. faecium* FC12 incubated at 20°C in the presence of different concentrations of plant derivatives: 1 mg/ml of phenolic extracts (PE) of *J. oxycedrus* needles or *R. fruticosus* leaves, 1 mg/ml of EO of *J. oxycedrus* needles and 0.75 mg/l of EO of *R. fruticosus* leaves. The experimental data obtained by plate counting are represented with points, while curves are the relative fitted models obtained with Gompertz equation.

Table 5.7 reports the parameters estimated for the models used, in which the maximum cell concentration attained, according to the models and some diagnostics of fitting, are also reported.

Table 5.7: *Ent. faecium* FC12 growth parameters in the presence of different plant derivatives (PE or EO), estimated by modelling the data from sampling (log cfu/ml) with the Gompertz equation. The maximum cell concentration attained according to the models and some diagnostics of fitting are also reported.

Samples	k	$A1^a$	$\mu_{max}1^a$	$\lambda1^a$	$A2$	$\mu_{max}2$	$\lambda2$	Max cell load	R	RMSE
Control	6.03	-	-	-	3.00	0.309	0.11	9.00	0.993	0.157
J. oxycedrus PE	5.92	-	-	-	2.11	0.069	24.14	8.11	0.994	0.187
R. fruticosus PE	6.08	-	-	-	2.89	0.324	0.08	8.89	0.986	0.377
J. oxycedrus EO	5.98	-1.30	-0.128	0.06	3.64	0.160	17.12	8.34	0.995	0.192
R. fruticosus EO	6.05	-1.18	-0.174	0.95	3.41	0.095	33.77	8.23	0.994	0.189

[a]: parameters estimated only for the double-peaked Gompertz model used when the initial cell concentration decreased before the growth started again

Ent. faecium FC12 cells in the control rapidly started their exponential phase, with the lag phase estimated length of 0.11 h, and reached the maximum cell concentration (approx. 9 log cfu/ml) in less than 24 h, with a maximum growth rate (μ_{max}) of 0.309 (log cfu/ml)/h. The Gompertz parameters estimated for the control and the sample containing *R. fruticosus* PE were similar and the consequent models were almost completely superimposable, indicating the absence of any antimicrobial effect of this extract. However, the PE of *J. oxycedrus* determined a marked increase of the lag phase of approx. 24 h, a reduced μ_{max} value (0.069 (log cfu/ml)/h) and a maximum growth of 8.11 log cfu/ml, resulting in almost 1 log unit lower than control.

In previous trials (Paragraph 5.1.3.2), the same PE were more effective in reducing growth kinetics of *List. monocytogenes* when used at the 50% of the MIC. However, in this case a relevant inhibition of *Ent. faecium* was obtained only with *J. oxycedrus* PE, which contains not only high amounts of vanillic acid (10.51 mg/l), apigenin (7.66 mg/l) and rutin (6.95 mg/l), but also gallic acid and p-hydroxybenzoic acid (Table 5.1). The antimicrobial potential of the molecules found in these PE was reviewed by Oulahal and Degraeve (2022). Rutin, apigenin and gallic acid showed good antimicrobial activity against *Ent. faecalis* (Gutiérrez-Fernández et al., 2013; Adamczak et al., 2020). Taviano et al. (2013) observed a remarkable antimicrobial activity against *Enterococcus hirae* of methanolic extracts of *J. oxycedrus* berries, containing rutin, apigenin, cupressoflavone, ametoflavone and methyl-biflavone among the others.

The *R. fruticosus* PE was rich in rutin, chlorogenic acid, astrignin, and caffeic acid (Table 5.1), but showed no antimicrobial activity against *Ent. faecium* under the conditions adopted in this trial. Interesting, antimicrobial effects on *Ent. faecalis* were demonstrated by extract from *R. fruticosus* rich in ellagic acid, which was absent in the PE used in this analysis (Ekrikava et al., 2021). Moreover, *Ent. fecalis* showed high susceptibility to quercetin in combination with gallic acid and chlorogenic acid, while insufficient effects were obtained with rutin (Jobim et al., 2014).

The mechanism of action responsible for the antimicrobial activity of these molecules is not completely clear. Literature suggests that some compounds (*e.g.* quercetin, gallic acid and chlorogenic acid) could induce an imbalance of the redox potential, while others (*e.g.* apigenin and rutin) could interfere with enzymes of DNA replisome or perturbate membrane permeability and functions (Jobim et al., 2014). Recently, Xu et al. (2021) observed a depolarisation and permeabilization of the membrane and an alteration of the intracellular enzymatic activities in *Staph. aureus* when treated with a phenolic extract from *Taraxacum officinale* that contained rutin, caffeic acid, and chlorogenic acid.

Both EO determined an initial decrease in *Ent. faecium* culturability. In the sample containing *J. oxycedrus* EO, a reduction of more than 1 log unit of the initial population was observed after 10 h, followed by a cell load increase ($\lambda 2$ estimated at 17.12 h). However, in this second phase, the μ_{max} (0.160 (log cfu/ml)/h) and the final cell concentration in the stationary phase (8.34 log cfu/ml) were lower if compared to the control.

The main components of the *J. oxycedrus* EO used in this trial were terpenes and terpenoids. Limonene (13.6%), α-pinene (10.8%) and manoyl oxide (8.4%) were the main constituents, followed by 3-carene, α-curcumene and 4(15),5-muuroladiene (Table 5.2). Recently, an EO from *Juniperus phoenicea*, whose main constituents were α-pinene, δ-3-carene and β-caryophyllene, showed good antimicrobial activity against a strain of *Ent. faecalis*, while Najar et al. (2020)

reported a variable inhibitory effect of *J. oxycedrus* EO, depending on the geographical area. Similarly, *Ent. faecalis* was the most susceptible species to an EO from *Juniperus horizontalis* leaves, containing p-cymene and linalool as the major constituents (Eryiğit et al., 2014). However, no studies regarding the effects of specific EO constituents on enterococci are available.

J. oxycedrus EO and *R. fruticosus* leaves EO reduced the initial culturability of *Ent. faecium* FC12 although the successive growth started later (λ2 estimated at 33.77 h) and slowly (μ_{max} 0.095 (log cfu/ml)/h), with a final predicted cell concentration that reached 8.23 log cfu/ml. *R. fruticosus* EO major constituents were geraniol (13.7%), phytol (4.9%), β-citronellol (4.6%), linalool (4.1%) and β-ionone (3.7%). In addition, the EO contained methyl salicylate (1.3%) and citral (2.4%) (Table 5.3). Even if many of these compounds were studied in relation to their antimicrobial activity, no data are reported concerning their effect on enterococci (Álvarez-Martínez et al., 2021).

Antimicrobial effects of terpenes, terpenoids and phenylpropanoids is strictly dependent on their chemical structure and the presence of specific functional groups. For this reason, the mechanisms of action can be extremely different from one compound to another. However, the first requirement for EO active components relies on the possibility to solubilize in cell membranes which, in many cases, are the major target of their bacteriostatic or bactericidal effects (Álvarez-Martínez et al., 2021; da Silva et al., 2021; Gottardi et al., 2016; Reyes-Jurado et al., 2020).

5.2.3.2 Determination of biogenic amines production

Ent. faecium FC12 was already studied for its tyrosine decarboxylase activity (Bargossi et al., 2015a,b). For this reason, the formation of tyramine was monitored during growth with or without the PE and EO (Figure 5.4).

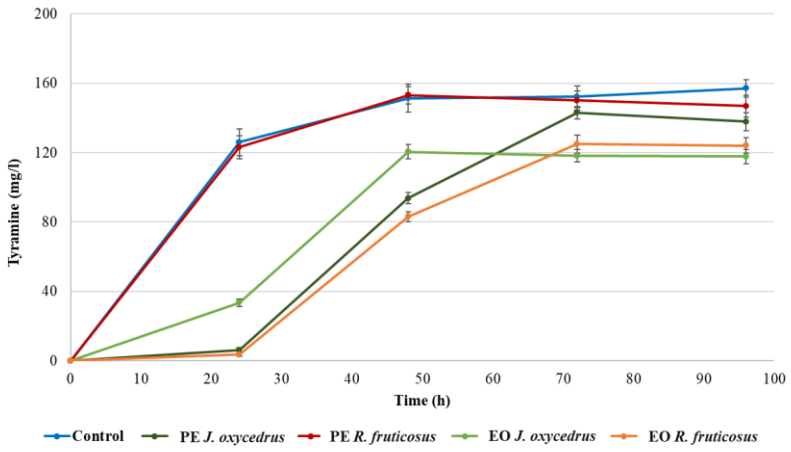


Figure 5.4: Tyramine production by *Ent. faecium* FC12 during incubation at 20°C in the presence of different concentrations of plant derivatives. The data are the mean of three independent samples and standard deviations are reported.

As already observed for the growth curves, the control and sample containing *R. fruticosus* PE showed almost the same behaviour. In fact, in both cases, a relevant amount of tyramine (approx. 120 mg/l) was present after 24 h, when the cells had already reached their stationary phase. The concentration slightly increased after 48 h (about 150 mg/l) and then remained constant.

The *J. oxycedrus* PE delayed the tyramine formation, with only 6 mg/l detected after 24 h incubation. This concentration increased together with the cell growth, reaching an amount comparable with the control and sample containing *R. fruticosus* PE, after 96 h. When *J. oxycedrus* EO was added to the growth medium, tyramine concentration was 33.5 mg/l after 24 h of incubation, with an estimated cell concentration of 5.8 log cfu/ml). Then, it increased up to 118.2 mg/l after 48 h, when the maximum cell load of 8.1 log cfu/ml was reached, and remained stable for the subsequent 48 h. A similar maximum concentration was obtained in the samples containing *R. fruticosus* EO, which, however, showed a slower accumulation kinetic, following the growth curve reported in Figure 5.3.

It is known that, when tyrosine is depleted, the tyrosine decarboxylase of enterococci can use another aromatic amino acid as substrate, *i.e.* phenylalanine, producing 2-phenylethylamine (Marcobal et al., 2012; Bargossi et al., 2015b, 2017). For this reason, this amine was also monitored (Figure 5.5).

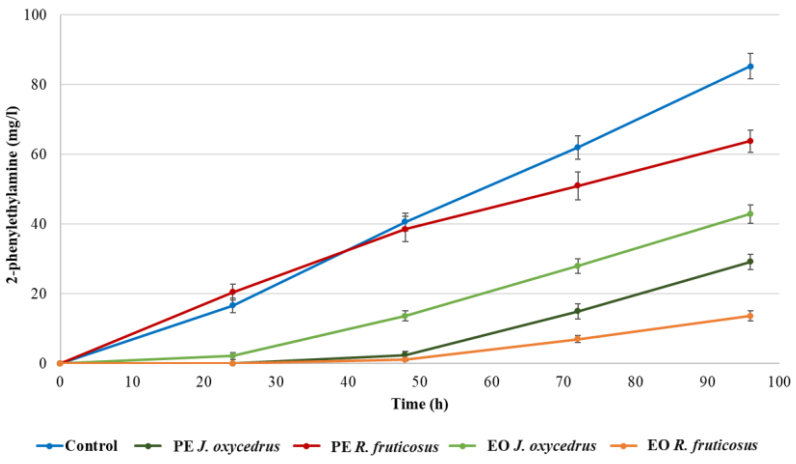


Figure 5.5: 2-phenylethylamine production by *Ent. faecium* FC12 during incubation at 20°C in the presence of different concentrations of plant derivatives. The data are the mean of three independent samples and standard deviations are reported.

As expected, it was produced only after tyramine concentration reached its maximum. In the control, 85.3 mg/l of 2-phenylethylamine were found at the end of incubation (96 h). Despite the growth data and tyramine production, in sample containing *R. fruticosus* PE, 2-phenylethylamine accumulation had a trend similar to the control during the first 48 h of incubation, while lower

values were measured after 96 h (63.8 mg/l). Conversely, the presence of *J. oxycedrus* EO delayed the production of this amine after 48 h, when tyramine reached its maximum concentration. Even *J. oxycedrus* PE and *R. fruticosus* EO showed the same trend, with the latter EO determining the lowest accumulation of 2-phenylethylamine after 96 h, confirming its antimicrobial effect.

As already observed, detectable tyramine accumulation by *Ent. faecium* started in the last part of the exponential growth phase, while 2-phenylethylamine was produced by cells in the stationary phase following the depletion of tyrosine (Bargossi et al., 2017). The availability of precursors depends on the media composition. Previous experiments showed that *Ent. faecium* FC12 grown in the same medium (BHI) under optimal conditions (37°C without antimicrobials) determined a final ratio of 3:1 between 2-phenylethylamine and tyramine (Bargossi et al., 2015b). In this case, after 96 h of incubation, its concentration had still an increasing trend in all the conditions, including the control.

5.2.3.3 Effect of plant derivatives on cell viability and culturability

With the aim to evaluate the impact of EO and PE on *Ent. faecalis* FC12 viability, a flow cytometry analysis was performed and the data obtained after 0, 24, 48, and 72 h of incubation at 20°C were compared with those deriving from plate counting. In particular, the total cell number, expressed as log of cells stained with SYBR Green I independently of their physiological state, was compared with cell culturability, expressed as log cfu/ml as predicted by the models. In addition, for each condition and sampling time, the percentages of viable, injured, and dead cells detected after the dual staining with SYBR-Green I and propidium iodide were also reported (Figure 5.6).

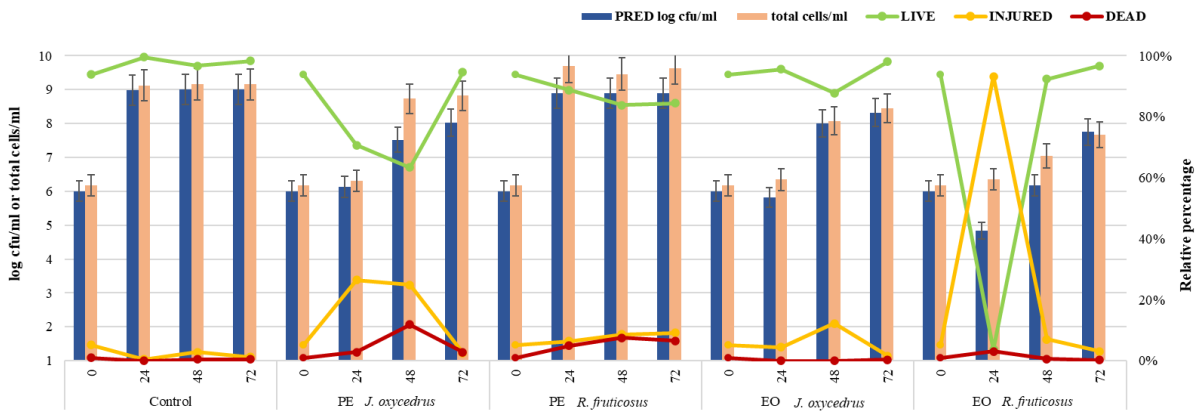


Figure 5.6: Comparison between the data of cell culturability (expressed as log cfu/ml predicted by the models) and the total cells detected by flow cytometry analysis (log total cells/ml) of *Ent. faecium* FC12 grown in the presence of different plant derivatives (PE or EO of *J. oxycedrus* and *R. fruticosus*) after 24, 48 and 72 h of incubation at 20°C. For this latter, also the relative percentages of live, injured, and dead cells for each condition (as green, yellow and red lines, respectively) are reported. The presence of an asterisk indicates significant differences between the data obtained through sampling and flow cytometry analysis.

In the control, culturability (plate counting) and total cells (flow cytometry analysis) did not show significant differences. The viable cells were the 94% at time 0 and ranged between 96 and 99% during incubation. Similar values were obtained in the sample containing *R. fruticosus* PE, although a slight decrease in viable cells was observed after 24 and 48 h. With the addition of *J. oxycedrus* PE, the number of culturable and total cells at 24 h did not increase compared to the initial concentration. Even though both values were not significantly different (6.13 log cfu/ml *vs.* 6.30 total cells/ml), viable cells represented the 71% of total population (corresponding to a concentration of 6.15 log cells/ml), while injured cells were 26%. After 48 h, the culturable cells increased to 7.52 log cfu/ml, whereas the total cell number was 8.73 log total cells/ml. This difference can be related to the presence of viable but non-culturable state cells resulting from *J. oxycedrus* PE treatment that induced cell damage. This is supported by the fact that the percentage of cells recognised as live (*i.e.* with intact cell membrane) still decreased (63%, corresponding to 8.53 log live cells/ml) and dead cells increased (about 12% of the total population). At the end of incubation (72 h), total cells and culturable cells were again not significantly different and a higher number of viable cells (95%) was reached. Therefore, *J. oxycedrus* PE induced temporary cell damage resulting in a slower growth kinetic, namely an increase in lag phase duration and a decrease in growth rate. The same effect with the same PE was already reported in the precious trial performed against *List. monocytogenes* (Paragraph 5.1.3.2).

Data concerning the *J. oxycedrus* EO showed comparable numbers of culturable and total cells during all the incubation. The percentage of live cells ranged from 87.8% to 98.0% log cfu/ml and the values of viability detected by flow cytometry analysis (log live cells/ml) were substantially the same, indicating an exponential growth between 48 and 72 h.

A different behaviour was observed in the presence of *R. fruticosus* EO. The number of total cells detected by flow cytometer did not significantly change between time 0 h and time 24 h (6.17 *vs*. 6.35 total cells/ml). However, the cells recognised as live corresponded to 3.7% of the total population, while 93.2% resulted as injured. Thus, live cells were 4.92 log cells/ml, a value comparable with the cells detected by sampling (4.83 log cfu/ml). After 48 h, almost all the cells recovered (92.3%), with 7.02 log live cells/ml compared to the predicted cell concentration that was lower (6.17 log cfu/ml). In fact, at this time, a great part of live cells resulted unculturable with the traditional plate counting. After 72 h, the data from flow cytometer and plate counting were similar, being 7.66 log live cells/ml and 7.75 log cfu/ml, respectively. The effect of *R. fruticosus* EO on *Ent. faecium* during the first hours of incubation was in line with the results previously described regarding *List. monocytogenes*, indicating the ability of this EO to induce relevant cell damage (Paragraph 5.2.3.2). However, while in their work damages were lethal up to 48 h (more than 99%

of dead cells), those observed in *Ent. faecium* cells were transient since the microorganism was able to recover within the same time frame. Although the differences observed could depend on the specific constituents of the extracts and their interaction with the target microorganism, the exact mechanism of cell recovery activated by *Ent. faecium* upon exposure to these plant derivatives is still unknown and requires further studies.

5.2.4 Conclusions

Plant PE and EO can have an important role in limiting the growth of spoilage, pathogenic or toxin producer microorganisms in foods. In this part of the thesis, the response of the tyraminogenic strain *Ent. faecium* FC12 to sub-lethal concentrations of *J. oxycedrus* needles and *R. fruticosus* leaves plant derivatives was evaluated, in terms of cell culturability, cell viability, tyramine and 2-phenylethylamine production. The tested PE were less effective in limiting *Ent. faecium* kinetics and BA production than the corresponding EO. In particular, *R. fruticosus* PE did not show any effect, while *J. oxycedrus* PE reduced the growth dynamic of the strain and the resulting tyramine formation. According to flow cytometry analysis, *J. oxycedrus* PE increased the dead or injured cells between 24 and 48 h of incubation. Regarding two EO, both determined an initial decrease in culturability. In particular, *R. fruticosus* EO was the most effective since many cells were injured already after 24 h. Then, they were able to revert to a metabolic active state with lower growth rates and a reduced production of tyramine. According to these data, some of the tested plant extracts showed an interesting antimicrobial potential against *Ent. faecium*. Although the discrepancies between cell culturability and viability must be carefully considered to avoid underestimations of the effective metabolic responses to stress conditions, these “green” solutions can be considered as promising candidates for the microbial stabilisation and shelf-life extension of foods. Indeed, further studies are in progress, and they want to assess the suitability of these approaches to control enterococci, as well as other spoilage or pathogen microorganisms, in animal-based foods (*e.g.* meat products).

Data published as:

Montanari, C., **Barbieri, F.**, Lorenzini, S., Gottardi, D., Šimat4, V., Özogul, F., Gardini, F., Tabanelli, G.* (2023). Survival, growth and biogenic amine production of *Enterococcus faecium* FC12 in response to extracts and essential oils of *Rubus fruticosus* and *Juniperus oxycedrus*. *Frontiers in Nutrition*, 9, 1092172. doi: 10.3389/fnut.2022.1092172.

Chapter 6:

Increasing of bio-active compounds availability in vegetal matrices fermented by LAB strains

6.1 Impact of LAB fermentation on phenolic compounds and antioxidant activity of avocado leaf extracts

6.1.1 Introduction

Avocado (*Persea americana* Mill., Lauraceae) has the largest production areas concentrated in Central and South America, in particular in Mexico, but also in United States, Indonesia, Kenya and South Africa (Mora-Sand et al., 2021; Sommaruga and Eldridge, 2021). The most traded avocado is the *Hass* variety, that was first introduced around the 1960s in Michoacán (Mexico) (Ramírez-Mejía et al., 2022). In 2021, the Food and Agriculture Organization of the United Nations calculated a world avocado production of 8.69 million tons (https://www.fao.org), with respect to the 7.0 million tons reported in 2019 (Zafar and Sidhu, 2018). The intensification in the production and consumption of avocado fruit led to a consequent increase in the amount of agricultural food wastes, becoming a serious environmental problem, as well as economy and society concern (Capanoglu et al., 2022). Trujillo et al. (2020) demonstrated the antioxidant properties of avocado fruit and these results suggest that the recovery of these by-products could be a suitable solution to these concerns and to develop a sustainable economy growth (Mora-Sand et al., 2021). Different approaches are available for the recovery of bio-active compounds from wastes as potential ingredients to be added in the production of functional foods (Jimenez et al., 2021).

During harvest, avocado leaves are discarded without industry application, although in plant leaves phenolic compounds were demonstrated to be found in high concentration. Their presence depends on genetic factor, environmental conditions, geographic location and physiological variations (Martins et al., 2011). In several studies the extracts obtained from avocado leaves were described to contain bio-active compounds, such as alkaloids, triterpenoids, saponins, carbohydrates, fatty acids and polyphenols (Nathaniel et al., 2015; Park et al., 2019; Yamassaki et al., 2018).

Phenolic compounds are characterised by redox properties, that can neutralise free radicals, contributing to an amelioration of some degenerative diseases associated to oxidative stress (Rojas-García et al., 2022). Moreover, these bio-active compounds have been also associated to antibacterial or antifungal activities. In their study, Castro-López et al. (2019) reported that hydroalcoholic leaf extracts of seven Mexican cultivars of *Per. americana* var. *drymifolia* showed a strong antioxidant activity, assessed by DPPH and ABTS assays, while purified phenolic fractions of avocado leaf extracts showed a concentration-dependent antibacterial effect (Solís-Salas et al., 2021). However, functional activities of these compounds depend on their chemical structure and bioavailability. In the human gut, dietary phenolics are transformed by fermentative microbiota,

which plays an important role in their absorption and in the modulation of their functional properties with benefits in consumers health (Selma et al., 2009).

In food industry, LAB fermentation is used to enhance the nutritional quality of foods, increasing protein digestibility, mineral availability and the release of peptides and amino acids (Verni et al., 2019), but also to improve food safety through their antimicrobial and antioxidant activities (Curiel et al., 2015; Pontonio et al., 2019). In particular, these bacteria can be applied in vegetable fermentation, due to their good adaptation in this environment (Filannino et al., 2018; Wang et al., 2021). The capability of LAB to process plant substances is species and strain-specific and it is linked to the presence of enzymes (*e.g.* glucosidase, amylase, cellulase, tannase, chitinase and lipase) that can hydrolyse and break down compounds to release functional substances during fermentation (Muñoz et al., 2017). On the other hand, LAB metabolism and viability can be affected by several factors, such as lacking fermentable carbohydrates or the presence of inhibitory factors (*i.e.* phenol compounds) (Filannino et al., 2015). For this reason, the tolerance to high levels of phenols is a required feature for LAB strains selected to transform plant matrices (Filannino et al., 2018).

This part of the PhD thesis, carried out at the Department of Nutrition and Food Sciences (University of Granada) under the supervision of Professor Vito Verardo, was aimed to evaluate the effect of the fermentation exerted by same selected LABs on avocado leaves as a strategy to obtain extracts enriched in phenolic compounds. In particular, the total phenolic content and the antioxidant activity, analysed by DPPH and FRAP assays, were determined from hydroalcoholic extracts obtained from fermented samples.

6.1.2 Materials and methods

6.1.2.1 Avocado leaves collection and LAB strains used

Avocado leaves of *Hass* variety were collected from Salobreña (Spain) in April 2022. The fresh leaves were air dried at room temperature in the dark. Then, they were minced and sieved to 100 µm particle size. The samples were stored at -18°C until the analyses.

To guide the fermentation of avocado leaves, LAB strains of the Spanish Collection of Type Cultures (CECT) were selected. In particular, they belonged from the species *Pediococcus acidilactici* (5765T and 98 strains), *Pediococcus pentosaceus* (4695T and 923 strains), *Leuconostoc mesenteroides* subsp. *mesenteroides* (219T strain), *Levilactobacillus brevis* (4121T and 5354 strains), *Lactiplantibacillus plantarum* subsp. *plantarum* (748T and 9567, formerly C4, strains). Strains were pre-cultivated in MRS broth, at 30ºC for 24 h, and stocks were stored in glycerol at -20ºC until the analyses.

6.1.2.2 Avocado leaves fermentation

In order to carry out avocado leaves' fermentation, 1 g of dried samples were covered by 8 ml of sterile water, previously heated to 90ºC. After mixing and cooling, the mixture was supplemented with 1 ml of sterile medium, formulated with glucose and yeast extract to obtain a concentration of 0.4% w/v of each one. LAB inoculum was added in a final load of about 7.5 log cfu/g. LAB counting (on MRS agar plates, according to Paragraph 3.1.2.3) and pH values (Paragraph 3.1.2.2) were monitored at 0, 24, 48, 72 and 96 h of incubation at 30°C. At the end of incubations, samples were stored at -20ºC and then freeze-dried for further determinations. Each analysis was performed in triplicate.

6.1.2.3 Polar compound extraction

Polar compound extraction was performed with 0.2 g of freeze-dried avocado fermented leaves powder dissolved in 6 ml solution of ethanol/water (80:20 v/v). The mixture was treated in an ultrasonic bath for 15 min, and then it was centrifuged for 10 min at 9000 rpm. The extracting procedure was repeated twice. All the supernatants were collected, evaporated and reconstituted in 1 ml of methanol/water (50:50 v/v). The final extracts were filtered with regenerated cellulose filters 0.2 µm (Millipore, Bedford, USA) and stored at -18°C until further analyses.

6.1.2.4 Determination of Folin-Ciocalteu reacting substances

A first screening was performed by using Folin-Ciocalteu spectrophotometric method, in order to determine the total Folin-Ciocalteu reacting substances (FCRS) in all samples of fermented avocado leaves (Lamuela-Raventós, 2017). Briefly, 500 µl of the Folin-Ciocalteu reagent was added to 100 µl of extract. Then, 6 ml of bi-distilled water were added and the solution was agitated for 1 min. After that, 2 ml of 15% (w/v) Na_2CO_3 and again bi-distilled water, to reach a final volume of 10 ml, were added. The samples were left in darkness for 2 h and the measures were carried out at 750 nm with a UV-visible spectrophotometer (Spectrophotometer 300 Array, UV-Vis, single beam, Shimadzu, Duisburg, Germany). Calibration curve was carried out with gallic acid from 1 to 1000 ppm. Results are expressed as mg gallic acid equivalents (GAE)/g dry weight (d.w.).

6.1.2.5 Determination of antioxidant activity: DPPH and FRAP assays

Antioxidant activity of avocado fermented leaves extracts was determined by DPPH and FRAP assays (Razola-Díaz et al., 2021, 2022). The measurements were performed using an UV-visible spectrophotometer (Spectrophotometer 300 Array, UV-Vis, single beam). In both assays,

Trolox was used as standard for the calibration curves and results were expressed in mg of Trolox equivalents (TE)/g of dry weight (d.w.).

6.1.2.6 Determination of polar compounds by HPLC-ESI-TOF-MS

Phenolic compounds present in the fermented and non-fermented avocado leaves extracts were analysed using an ACQUITY Ultra Performance Liquid Chromatography (UPLC) system, coupled with an electrospray ionization (ESI) source, operating in the negative-mode, and a mass detector time of flight (TOF) micro mass spectrometer (Waters Corporation, Milford, USA). Compounds of interest were separated with an ACQUITY UPLC BEH Shield RP18 column (1.7 μm, 2.1 × 100 mm; Waters Corporation), maintained at 40°C. Parameters and the gradient condition used followed the guidelines of Verni et al. (2020). Acidified water (1% of acetic acid) and acetonitrile were used as phase A and B, respectively. Analyses were performed in triplicate. The identification of the phenolic compounds was made with a mass accuracy score higher than 90%. Finally, to quantify the identified phenolic compounds, calibration curves of vanillic acid, chlorogenic acid, ferulic acid, quercetin, catechin and rutin were used.

6.1.2.7 Data processing

The data collected with HPLC-ESI-TOF-MS analysis were elaborated using MassLynx 4.1 software (Waters Corporation). Significative differences were defined by using Statistica 8.0 software (StatSoft Inc.). Moreover, to perform the other statistical analyses MetaboAnalyst 5.0 was employed.

6.1.3 Results and discussion

6.1.3.1 Detection of LAB strains during avocado leaves fermentation

Selected LAB strains were added to guide the fermentation of dried avocado leaves at 30°C. This microbial population was enumerated through sampling at 0, 24, 48, 72 and 96 h on MRS agar plates. Results, reported in Table 6.1, showed that avocado leaves did not support the growth of most of the inoculated microorganisms. In general, LAB strains decreased in the first hours of incubation and this behaviour was observed during the entire fermentation period. On the other hand, in samples fermented by *Ltp. plantarum* 748T and *Ltp. plantarum* 9567 strains a growth during the fermentation was detected, reaching a maximum cell concentration of about 8.4 log cfu/g.

Table 6.1: LAB counts (log cfu/g) during avocado leaves fermentation. Standard deviation is also reported.

LAB strains	0 h	24 h	48 h	72 h	96 h
P. acidilactici 5765T	7.93 ± 0.23	7.86 ± 0.31	7.68 ± 0.25	7.75 ± 0.16	7.20 ± 0.27
P. acidilactici 98	7.81 ± 0.28	7.47 ± 0.34	7.31 ± 0.35	6.66 ± 0.24	6.36 ± 0.16
P. pentosaceus 4695T	7.25 ± 0.15	6.50 ± 0.23	6.36 ± 0.36	5.08 ± 0.34	4.25 ± 0.25
P. pentosaceus 923	7.72 ± 0.21	6.69 ± 0.22	6.25 ± 0.16	5.47 ± 0.24	5.01 ± 0.32
Leuc. mesenteroides 219T	7.23 ± 0.18	6.26 ± 0.19	5.37 ± 0.25	5.07 ± 0.16	5.64 ± 0.25
Lev. brevis 4121T	7.45 ± 0.26	6.72 ± 0.23	6.63 ± 0.22	6.09 ± 0.30	5.36 ± 0.18
Lev. brevis 5354	7.38 ± 0.24	6.24 ± 0.35	5.48 ± 0.36	5.21 ± 0.28	5.13 ± 0.24
Ltp. plantarum 748T	7.94 ± 0.14	8.44 ± 0.11	8.41 ± 0.15	8.30 ± 0.18	8.15 ± 0.10
Ltp. plantarum 9567	7.98 ± 0.22	7.79 ± 0.27	8.45 ± 0.12	8.48 ± 0.10	8.20 ± 0.14

These results were confirmed also by the pH values. The initial pH was about 6 and, after 24 h of incubation, *Ltp. plantarum* 748T strain caused the acidification of the samples, reaching value of 3.93, that remained constant until the end of incubation. In the case of *Ltp. plantarum* 9567 strain, a pH reduction up to 4 was observed after 48 h of incubation (Figure 6.1). Regarding the other strains, pH was maintained similar to the initial value.

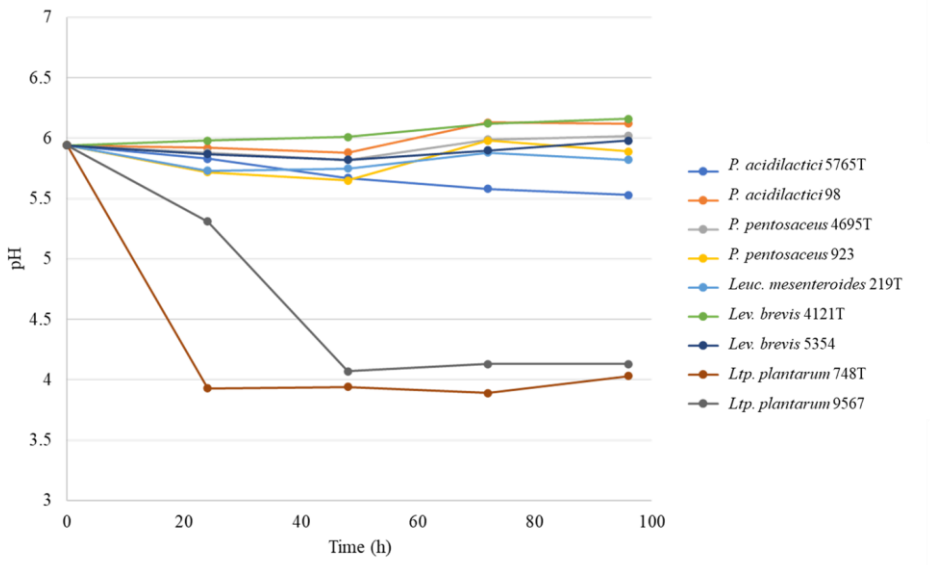


Figure 6.1: pH values detected during fermentation of avocado leaves by LAB strains.

Avocado leaves composition could affect the growth kinetics of these LAB strains. In particular, phenolics compounds can be a hurdle to their viability and metabolism (Leonard et al., 2021). Moreover, this vegetal matrix is characterised by glycosides, alkaloids, tannins, saponins, flavonoids, terpenoids and steroids, that represent a potential source of antimicrobial molecules (García-Rodríguez et al., 2016; Park et al., 2019; Solís-Salas et al., 2021). Nevertheless, high tolerance to phenolic compounds is found in LAB microbial group, especially in Lactobacilli strains isolated from fermented products, characterised by a high content of phenolic compounds

(Ghabbour et al., 2011). In this context, *Ltp. plantarum* has been widely studied for its adaptation to plant habits and capability to metabolise phenolics and it is used as starter in food fermentation (Filannino et al., 2015).

6.1.3.2 Folin-Ciocalteu reacting substances content and antioxidant activity of the fermented avocado leaves

Avocado leaves were previously treated with hot water, in order to eliminate contaminants that could affect the fermentation process. It was reported that phenolic compounds content or antioxidant activity of the extracts were not affected by their exposure to high temperatures until 8 h (Yamassaki et al., 2017).

All fermented avocado leaves samples, collected at different incubation times (24, 48, 72 and 96 h), were analysed to evaluate their content in Folin-Ciocalteu reacting substances (FCRS) and antioxidant activity. The results are reported in Table 6.2.

An increase of the FCRS content has been detected after the fermentation of some LAB strains, respect to the control (26.90 mg GAE/g d.w.). In particular, after 24 h FCRS values were detected between 27.70 mg GAE/g d.w. and 29.56 mg GAE/g d.w. in samples fermented by *P. acidilactici* 5765T, *P. pentosaceus* 4695T, *Lev. brevis* 5354 and *Ltp. plantarum* 9567, but the highest value was observed in *Ltp. plantarum* 748T sample after 48 h (30.72 mg GAE/g d.w.). In the other samples, up to the end of incubation, this parameter was always significantly lower respect to the control.

Otherwise, comparing the control sample at initial time, antioxidant activity was lower in all samples during fermentation, according to data obtained with DPPH and FRAP assays. As reported in Table 6.2, the highest antioxidant activity was measured after 24 h and 48 h of incubation. These values ranged from 44.53 mg TE/g d.w.to 51.32 mg TE/g d.w. (*P. acidilactici* 5765T) in DPPH analysis and 78.50 mg TE/g d.w.to 96.61 mg TE/g d.w. (*Ltp. plantarum* 748T) according to FRAP test. Filannino et al. (2020) reported that, in avocado puree, the fermentation guided by *Ltp. plantarum* resulted in a high level of total free amino acids and a marked increase of antioxidant activity.

The FCRS content was related to the antioxidant activity. A significant positive correlation ($p < 0.05$) was found between total phenolic content and DPPH (r = 0.7857) and FRAP (r = 0.8069) assays. Likewise, DPPH assay showed a significant positive correlation with FRAP assay (r = 0.6107).

Table 6.2: Folin-Ciocalteu reacting substances content and antioxidant activity of all fermented avocado leaves samples. Results are expressed as average and standard deviation is reported.

Microorganisms	Time (h)	FCRS (mg GAE/g d.w.)	Antioxidant activity (mg TE/g d.w.)	
			DPPH	FRAP
Control	0	26.90 ± 0.04	53.88 ± 0.58	92.10 ± 1.26
P. acidilactici 5765T	24	29.56 ± 0.37	51.32 ± 0.37	78.50 ± 1.35
	48	25.87 ± 0.08	50.01 ± 0.23	70.31 ± 1.19
	72	24.49 ± 0.09	42.85 ± 0.27	50.34 ± 0.84
	96	22.80 ± 0.05	45.45 ± 0.37	71.12 ± 1.20
P. acidilactici 98	24	17.77 ± 0.01	28.90 ± 0.07	53.59 ± 0.90
	48	20.10 ± 0.04	33.09 ± 0.07	56.66 ± 0.96
	72	18.35 ± 0.53	32.11 ± 1.28	72.28 ± 1.21
	96	18.58 ± 0.21	31.33 ± 0.36	64.51 ± 1.08
P. pentosaceus 4695T	24	27.70 ± 0.20	50.04 ± 0.56	93.33 ± 1.57
	48	21.49 ± 0.24	48.03 ± 0.55	82.79 ± 1.41
	72	21.22 ± 0.16	36.45 ± 0.41	65.64 ± 1.11
	96	17.46 ± 0.13	36.63 ± 0.94	53.82 ± 0.93
P. pentosaceus 923	24	20.21 ± 0.02	42.56 ± 0.74	62.30 ± 1.03
	48	21.60 ± 0.08	41.38 ± 0.28	68.89 ± 1.14
	72	23.41 ± 0.21	48.79 ± 0.28	78.61 ± 1.35
	96	23.53 ± 0.59	49.70 ± 0.27	83.41 ± 1.41
Leuc. mesenteroides 219T	24	20.49 ± 0.43	47.47 ± 0.32	72.51 ± 1.20
	48	18.94 ± 0.25	43.39 ± 1.09	64.97 ± 1.08
	72	20.08 ± 0.01	35.38 ± 0.29	62.42 ± 1.04
	96	18.88 ± 0.11	30.79 ± 0.50	57.49 ± 1.00
Lev. brevis 4121T	24	20.06 ± 0.16	35.68 ± 0.52	62.31 ± 1.04
	48	19.85 ± 0.18	33.17 ± 0.01	58.29 ± 0.99
	72	17.83 ± 0.19	30.32 ± 0.70	58.53 ± 0.99
	96	19.31 ± 0.18	29.23 ± 0.33	60.57 ± 1.02
Lev. brevis 5354	24	29.39 ± 0.60	47.20 ± 1.54	91.58 ± 1.53
	48	27.62 ± 0.50	43.78 ± 1.27	86.34 ± 1.49
	72	25.67 ± 0.42	40.29 ± 1.36	81.48 ± 1.39
	96	19.87 ± 0.24	31.64 ± 0.89	63.43 ± 1.08
Ltp. plantarum 748T	24	21.98 ± 0.12	25.56 ± 0.46	71.58 ± 1.24
	48	30.72 ± 0.52	44.53 ± 1.00	96.61 ± 1.60
	72	30.07 ± 0.52	43.35 ± 0.92	92.01 ± 1.55
	96	26.62 ± 0.40	38.59 ± 1.04	85.14 ± 1.45
Ltp. plantarum 9567	24	29.09 ± 0.02	49.68 ± 0.78	90.38 ± 1.57
	48	27.65 ± 0.10	42.74 ± 0.39	93.23 ± 1.54
	72	28.50 ± 0.30	43.59 ± 0.66	89.72 ± 1.51
	96	27.08 ± 0.32	40.82 ± 0.75	89.10 ± 1.50

Based on the FCRS content and the antioxidant activity, samples fermented by *P. acidilactici* 5765T (24 h), *P. pentosaceus* 4695T (24 h), *Lev. brevis* 5354 (24 h), *Ltp. plantarum* 748T (48 h) and *Ltp. plantarum* 9567 (24 h) strains were selected to study their phenolic

composition. Conversely, other tested strains were discarded for the further analyses due to their not promising results detected.

6.1.3.3 Identification of polar compounds in fermented avocado leaves by HPLC-ESI-TOF-MS

The selected samples avocado leaves fermented samples and the control were characterised by HPLC-ESI-TOF-MS and a total of 48 polar compounds were identified (Table 6.3). In particular, 37 flavonoids, 7 phenolic acids and 4 other compounds were detected.

Table 6.3: Identified compounds in fermented and non-fermented avocado leaf by HPLC-ESI-TOF-MS. In blue are highlighted flavonoids compounds, in red phenolics acids and in black the other compounds.

Peaks	Time (min)	m/z experimental	m/z calculated	Error (ppm)	Score (%)	Molecular formula	Compounds
1	0.324	191.0554	191.0556	-1.0	100.00	$C_7H_{12}O_6$	Quinic acid isomer a
2	0.394	191.0549	191.0556	-3.7	100.00	$C_7H_{12}O_6$	Quinic acid siomer b
3	1.362	315.0703	315.0716	-4.1	99.97	$C_{13}H_{16}O_9$	Protocatechuic acid-4-glucoside
4	3.406	163.0398	163.0395	1.8	100.00	$C_9H_8O_3$	*p*-coumaric acid
5	4.688	353.0865	353.0873	-2.3	99.97	$C_{16}H_{18}O_9$	Chlorogenic acid
6	5.172	371.0977	371.0978	-0.3	95.46	$C_{16}H_{20}O_{10}$	Dihydroferulic acid 4-O-glucuronide
7	5.478	385.1153	385.1135	4.7	90.68	$C_{17}H_{22}O_{10}$	Sinapic acid-C-hexoside
8	5.644	577.1351	577.1346	0.9	99.30	$C_{30}H_{26}O_{12}$	Procyanidin dimer
9	6.016	221.044	221.045	-4.5	100.00	$C_{11}H_{10}O_5$	*p*-coumaroyl glycolic acid
10	7.138	165.0547	165.0552	-3.0	100.00	$C_9H_{10}O_3$	Dihydro-*p*-coumaric acid
11	7.199	865.1981	865.198	0.1	91.80	$C_{45}H_{38}O_{18}$	Procyanidin trimer
12	7.568	301.0342	301.0348	-2.0	96.02	$C_{15}H_{10}O_7$	Quercetin
13	7.903	625.1406	625.1405	0.2	98.96	$C_{27}H_{30}O_{17}$	Quercetin-diglucoside isomer a
14	7.973	739.1682	739.1663	2.6	90.78	$C_{39}H_{32}O_{15}$	Cinchonain-1a-(4beta->8)-catechin isomer a
15	8.048	625.1428	625.1405	3.7	99.53	$C_{27}H_{30}O_{17}$	Quercetin-diglucoside isomer b
16	8.147	451.1015	451.1029	-3.1	99.51	$C_{24}H_{20}O_9$	Cinchonain
17	8.263	739.1646	739.1663	-2.3	95.25	$C_{39}H_{32}O_{15}$	Cinchonain-1a-(4beta->8)-catechin isomer b
18	8.531	595.1297	595.1299	-0.3	94.55	$C_{26}H_{28}O_{16}$	Quercetin-3-O-arabinosyl-glucoside isomer a
19	8.668	595.1292	595.1299	-1.17	95.67	$C_{26}H_{28}O_{16}$	Quercetin-3-O-arabinosyl-glucoside isomer b
20	8.788	609.146	609.1456	0.7	99.01	$C_{27}H_{30}O_{16}$	Rutin isomer a
21	8.966	609.1456	609.1456	0.0	96.23	$C_{27}H_{30}O_{16}$	Rutin isomer b
22	9.07	595.1286	595.1299	-2.2	99.57	$C_{26}H_{28}O_{16}$	Quercetin-3-O-arabinosyl-glucoside isomer c
23	9.144	463.0862	463.0877	-3.2	94.20	$C_{21}H_{20}O_{12}$	Quercetin-3-glucoside isomer a
24	9.314	463.0866	463.0877	-2.4	99.16	$C_{21}H_{20}O_{12}$	Quercetin-3-glucoside isomer b
25	9.57	579.1331	579.135	-3.3	96.32	$C_{26}H_{28}O_{15}$	Luteolin 7-O-(2”-O-pentosyl)-hexoside isomer a
26	9.624	299.0183	299.0192	-3.0	98.09	$C_{15}H_8O_7$	Emodic acid isomer a
27	9.645	477.0653	477.0669	-3.4	99.22	$C_{21}H_{18}O_{13}$	Quercetin glucuronide
28	9.715	579.135	579.135	0.0	91.62	$C_{26}H_{28}O_{15}$	Luteolin 7-O-(2”-O-pentosyl)-hexoside isomer b
29	9.765	565.1204	565.1193	1.9	92.13	$C_{25}H_{26}O_{15}$	Quercetin 3-xilosyl-(1->2)-alpha-L-arabinopyranoside
30	9.901	447.0918	447.0927	-2.0	94.92	$C_{21}H_{20}O_{11}$	Kaempferol-O-hexoside isomer a
31	9.992	609.1456	609.1456	0.0	99.88	$C_{27}H_{30}O_{16}$	Rutin isomer c
32	10.06	299.0192	299.0192	0.0	99.87	$C_{15}H_8O_7$	Emodic acid isomer b
33	10.087	579.1343	579.135	-1.2	92.58	$C_{26}H_{28}O_{15}$	Luteolin 7-O-(2”-O-pentosyl)-hexoside isomer c

34	10.244	447.0915	447.0927	-2.7	93.19	$C_{21}H_{20}O_{11}$	Kaempferol-O-hexoside isomer b
35	10.311	505.096	505.0982	-4.4	96.80	$C_{23}H_{22}O_{13}$	Quercetin 3-O-glucose-6"-acetate
36	10.451	447.0913	447.0927	-3.1	93.55	$C_{21}H_{20}O_{11}$	Quercetin-O-deoxyhesoxide
37	10.493	461.0706	461.0779	-3.0	99.87	$C_{21}H_{18}O_{12}$	Kaempferol 3-glucuronide
38	10.567	549.124	549.1244	-0.7	92.41	$C_{25}H_{26}O_{14}$	Kaempferol 3,4'-dixyloside isomer b
39	10.77	549.1262	549.1244	3.3	93.06	$C_{25}H_{26}O_{14}$	Kaempferol 3,4'-dixyloside isomer a
40	10.845	579.136	579.135	1.7	94.44	$C_{26}H_{28}O_{15}$	Luteolin 7-O-(2"-O-pentosyl)-hexoside isomer d
41	10.944	593.1521	593.1506	2.5	99.34	$C_{27}H_{30}O_{15}$	Catechin diglucopyranoside
42	11.022	489.1032	489.1033	-0.2	93.58	$C_{23}H_{22}O_{12}$	Quercetin 3-O-acetyl-rhamnoside
43	11.266	531.1507	531.1503	0.8	99.48	$C_{26}H_{28}O_{12}$	Luteolin 7-[6-O-(2-methylbutyryl)-beta-glucoside] isomer a
44	11.291	489.1024	489.1033	-1.8	98.79	$C_{23}H_{22}O_{12}$	Kaempferol 3-O-acetyl-glucoside
45	11.374	531.15	531.1503	-0.6	94.94	$C_{26}H_{28}O_{12}$	Luteolin 7-[6-O-(2-methylbutyryl)-beta-glucoside] isomer b
46	11.618	431.0989	431.0978	2.6	93.85	$C_{21}H_{20}O_{10}$	Kaempferol-O-coumaroyl
47	11.808	563.1408	563.1401	1.2	94.47	$C_{26}H_{28}O_{14}$	Apigenin-C-hexoside-C-pentoside
48	12.313	285.0394	285.0399	-1.8	90.15	$C_{15}H_{10}O_6$	Kaempferol

Among flavonoids, flavan-3-ol is a well-known group of flavonoids usually found in avocado samples (Rincón-Hernández et al., 2011). In this case, catechin derivatives (procyanidin dimer, procyanidin trimer and catechin diglucopyranoside) were detected. Moreover, it was interesting to highlight the presence of cinchonain-1a-(4beta->8)-catechin isomer a and b, described in avocado leaves for the first time. In addition, quercetin was detected together with a total of 12 quercetin derivatives, identified in agreement with Castro-López et al. (2019), who previously described them in avocado leaves, except for quercetin 3-O-glucose-6"-acetate, quercetin 3-xilosyl-(1->2)-alpha-L-arabinopyranoside and quercetin 3-O-acetyl-rhamnoside. This latter was found it in berries by Mi et al. (2004). As reported by Castro-López et al. (2019), also apigenin-C-hexoside-C-pentoside, kaempferol, kaempferol-O-hexoside (isomer a and b), kaempferol 3-O-acetyl-glucoside and kaempferol-O-coumaroyl were identified. In other studies, the presence of kaempferol 3-glucuronide and kaempferol 3,4'-dixyloside (isomer a and b) kaempferol derivatives was detected (Nakane and Iwashina, 2015). Finally, among luteolin derivatives, luteolin 7-[6-O-(2-methylbutyryl)-beta-glucoside] (isomer a and b) were identified, in agreement to Xiong et al. (2020), who found them in sorghum.

Regarding phenolic acids, the presence of protocatechuic acid-4-glucoside, p-coumaric acid, chlorogenic acid and sinapic acid-C-hexoside were observed, as reported by López-Cobo et al. (2016), that previously identified them in avocado by-products. Besides, 2 coumaric acid derivatives, named p-coumaroyl glycolic acid and dihydro-p-coumaric acid, were detected for the first time in avocado leaves. These compounds had already been found in lentils seeds (Dueñas et al., 2007) and in olives (Boskou et al., 2006), respectively. In addition, a ferulic acid derivative (dihydroferulic acid 4-O-glucuronide) was identified in agreement with Hu et al. (2021), who found it in sweet cherries. In addition, a ferulic acid derivative (dihydroferulic acid 4-O-glucuronide) was

identified in agreement with Hu et al. (2021), who found it in sweet cherries. In a previous study about avocado wastes, Fan et al. (2022) reported the presence of a similar compound, named ferulic acid 4-O-glucoside, but it is the first time that this compound was found in avocado leaves.

Finally, the remaining compounds were identified as quinic acid isomer a and b (Figueroa et al., 2021; López-Cobo et al., 2016) and emodic acid, 2 isomers of trihydroxyanthraquinones.

6.1.3.4 Quantification of phenolic compounds by HPLC-ESI-TOF-MS and their biotransformation during fermentation in avocado leaves

The phenolic profile of each sample was also assessed to highlight some changes in the composition of these compounds, that could be dependent on LAB metabolisms and not only on their growth. For this reason, detected compounds were quantified with HPLC-ESI-TOF-MS and the results are summarised in Table 6.4.

Dada showed that fermentation processes caused modifications in phenolic profile of samples (Figure 6.2). Total phenolic compounds content increased in each fermented sample, except for *Lat. plantarum* 748T (3242.17 µg/g d.w.), with respect to the control (4163.45 µg/g d.w.). The higher quantity of these compounds was detected in sample fermented by *P. pentosaceus* 4695T (7003.05 µg/g d.w.), followed by *Lev. brevis* 5354, *P. acidilactici* 5765T and *Ltp. plantarum* 9567. This result depended on differences in the accumulation of flavonoids. In fact, the variation in the total quantified phenolic acids was less evident.

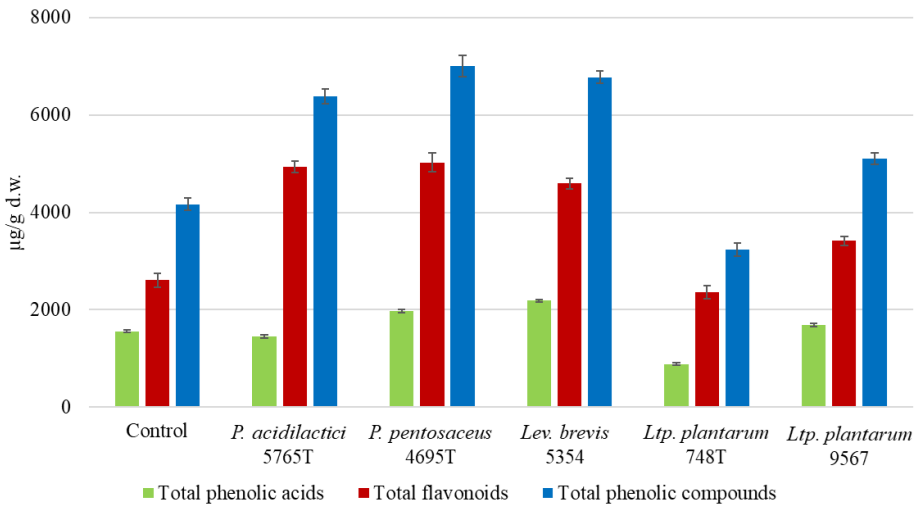


Figure 6.2: Phenolic profile in fermented avocado leaves samples, measured by HPLC-ESI-TOF-MS.

Table 6.4: Quantification of phenolic compounds of selected fermented avocado leaves samples. Only compounds beyond the limit of quantification (LOQ) are reported. Moreover, as a result of ANOVA test, different letters indicate significant differences among each compounds quantity in fermented samples with respect to the control.

Compounds (µg/g d.w.)	Control	*P. acidilactici* 5765T	*P. pentosaceus* 4695T	*Lev. brevis* 5354	*Ltp. plantarum* 748T	*Ltp. plantarum* 9567
Protocatechuic acid-4-glucoside	301.93 ± 2.66^{a}	235.18 ± 10.30^{b}	364.60 ± 7.40^{c}	401.68 ± 9.01^{d}	-^{e}*	384.61 ± 8.15^{d}
Chlorogenic acid	310.69 ± 5.89^{a}	37.84 ± 1.25^{b}	93.16 ± 0.66^{c}	194.14 ± 3.75^{d}	201.66 ± 3.66^{d}	95.63 ± 2.39^{c}
Dihydroferulic acid 4-O-glucuronide	516.74 ± 17.19^{a}	486.77 ± 13.16^{a}	766.37 ± 16.91^{b}	741.21 ± 6.43^{b}	419.82 ± 14.40^{c}	657.75 ± 6.57^{d}
Sinapic acid-C-hexoside	81.34 ± 1.54^{a}	115.99 ± 4.61^{b}	132.64 ± 2.93^{c}	129.81 ± 4.43^{c}	71.78 ± 1.16^{d}	120.71 ± 2.40^{b}
p-coumaric acid	147.97 ± 2.55^{a}	185.67 ± 5.25^{b}	200.73 ± 0.05^{c}	169.85 ± 2.25^{d}	-^{e}	140.29 ± 4.42^{a}
p-coumaroyl glycolic acid	24.05 ± 0.55^{a}	42.16 ± 1.94^{b}	56.81 ± 3.41^{c}	35.88 ± 2.36^{d}	-^{e}	30.27 ± 3.74^{d}
Dihydro-*p*-coumaric acid	121.23 ± 1.94^{a}	191.22 ± 4.13^{b}	230.99 ± 2.98^{c}	186.30 ± 2.94^{b}	165.49 ± 0.42^{d}	140.21 ± 5.34^{e}
Catechin diglucopyranoside	173.43 ± 2.46^{a}	174.76 ± 2.87^{a}	231.20 ± 14.79^{b}	168.59 ± 8.56^{ac}	156.56 ± 4.79^{c}	179.05 ± 3.94^{a}
Quercetin-diglucoside isomer a	214.69 ± 4.12^{a}	396.74 ± 5.37^{b}	370.98 ± 11.08^{c}	558.21 ± 4.90^{d}	277.86 ± 9.81^{e}	324.16 ± 5.66^{f}
Quercetin-diglucoside isomer b	98.53 ± 6.75^{a}	217.72 ± 10.52^{b}	170.00 ± 3.00^{c}	262.31 ± 3.76^{d}	124.72 ± 7.42^{e}	176.52 ± 4.49^{c}
Quercetin-3-O-arabinosyl-glucoside isomer a	269.02 ± 0.79^{a}	498.00 ± 79.73^{b}	394.58 ± 8.20^{c}	519.29 ± 8.56^{b}	270.03 ± 14.03^{a}	223.42 ± 10.62^{d}
Quercetin-3-O-arabinosyl-glucoside isomer b	126.63 ± 2.70^{a}	302.52 ± 10.79^{b}	284.07 ± 8.30^{b}	291.30 ± 7.83^{b}	103.57 ± 8.68^{c}	152.27 ± 10.32^{d}
Quercetin-3-glucoside isomer a	153.37 ± 3.88^{a}	248.31 ± 10.50^{b}	317.37 ± 16.93^{c}	272.94 ± 2.03^{d}	161.98 ± 5.52^{a}	191.52 ± 4.31^{e}
Quercetin-3-glucoside isomer b	221.66 ± 12.70^{a}	281.07 ± 16.50^{b}	291.79 ± 15.11^{b}	225.80 ± 8.41^{a}	15.13 ± 2.87^{c}	237.81 ± 4.13^{a}
Quercetin glucuronide	312.69 ± 11.19^{a}	244.54 ± 4.62^{b}	245.23 ± 9.88^{b}	310.17 ± 15.05^{a}	234.06 ± 6.78^{bc}	225.18 ± 3.68^{c}
Quercetin-O-deoxyhesoxide	102.90 ± 2.56^{a}	223.65 ± 8.65^{b}	233.55 ± 2.55^{b}	123.24 ± 1.33^{c}	75.82 ± 6.74^{d}	140.25 ± 3.27^{e}
Rutin isomer a	155.12 ± 10.53^{a}	348.26 ± 1.27^{b}	350.88 ± 3.37^{b}	324.72 ± 5.94^{c}	117.66 ± 8.92^{d}	219.94 ± 9.95^{e}
Rutin isomer b	94.67 ± 0.40^{a}	281.89 ± 0.47^{b}	257.55 ± 5.62^{c}	232.75 ± 6.29^{d}	54.63 ± 5.25^{e}	157.58 ± 7.31^{f}
Rutin isomer c	332.33 ± 13.44^{a}	557.92 ± 1.75^{b}	473.91 ± 13.52^{c}	498.48 ± 12.19^{c}	426.23 ± 10.28^{d}	527.58 ± 3.41^{e}
Luteolin 7-O-(2”-O-pentosyl)-hexoside isomer a	17.24 ± 3.20^{a}	184.54 ± 11.24^{b}	124.92 ± 9.70^{c}	88.77 ± 4.71^{de}	97.85 ± 5.37^{d}	82.09 ± 2.83^{e}
Luteolin 7-O-(2”-O-pentosyl)-hexoside isomer d	130.81 ± 3.40^{a}	146.00 ± 15.85^{ab}	181.60 ± 14.25^{b}	108.99 ± 3.12^{c}	69.94 ± 7.67^{d}	169.59 ± 1.49^{b}
Kaempferol	23.49 ± 0.99^{a}	32.04 ± 3.57^{b}	232.24 ± 25.93^{c}	26.53 ± 4.71^{ab}	-^{d}	2.82 ± 0.78^{e}
Kaempferol-O-hexoside isomer a	120.91 ± 7.55^{a}	309.41 ± 8.65^{b}	219.59 ± 9.05^{c}	258.68 ± 2.99^{d}	94.00 ± 6.02^{e}	148.73 ± 0.92^{f}
Kaempferol-O-hexoside isomer b	116.48 ± 6.57^{a}	258.40 ± 7.65^{b}	250.72 ± 11.78^{b}	177.49 ± 2.89^{c}	-^{d}	156.92 ± 9.50^{e}
Kaempferol 3-glucuronide	15.95 ± 2.62^{a}	154.26 ± 4.36^{b}	171.12 ± 1.78^{c}	101.26 ± 0.92^{d}	31.31 ± 5.45^{e}	40.93 ± 3.45^{e}
Kaempferol-O-coumaroyl	8.27 ± 1.39^{a}	38.94 ± 4.85^{b}	135.91 ± 3.08^{c}	8.34 ± 1.69^{a}	34.16 ± 6.92^{bd}	21.29 ± 7.20^{d}
Apigenin-C-hexoside-C-pentoside	22.80 ± 1.98^{a}	63.37 ± 8.24^{b}	137.46 ± 3.42^{c}	36.65 ± 4.60^{d}	-^{e}	17.34 ± 4.26^{a}
Total phenolic acids	**1557.08 ± 27.32^{a}**	**1445.14 ± 32.50^{b}**	**1976.73 ± 30.92^{c}**	**2185.23 ± 27.17^{d}**	**886.58 ± 19.65^{e}**	**1686.24 ± 27.02^{f}**
Total flavonoids	**2606.36 ± 137.24^{a}**	**4937.17 ± 113.27^{b}**	**5026.32 ± 190.43^{b}**	**4592.16 ± 105.17^{c}**	**2355.58 ± 133.52^{a}**	**3417.55 ± 90.51^{d}**
Total phenolic compounds	**4163.45 ± 124.56^{a}**	**6382.32 ± 145.78^{b}**	**7003.05 ± 221.35^{c}**	**6777.39 ± 132.34^{d}**	**3242.17 ± 133.17^{e}**	**5103.78 ± 117.53^{f}**

*: limit of quantification

The observed differences could be attributed to the degradation and biotransformation of food phenolic compounds by LAB tannase, amylase, esterase, β-glucosidase, phenolic acid decarboxylase (PAD), reductase and benzyl alcohol dehydrogenase enzymes (Muñoz et al., 2017). Hydroxycinnamic acids, such as caffeic, *p*-coumaric and ferulic acids, can be reduced into dihydrocaffeic, phloretic and dihydroferulic acids, respectively, or decarboxylated into vinyl derivatives by a phenolic acid decarboxylase enzyme (PAD), and subsequently reduced into ethyl derivatives (Filannino et al., 2014).

While *p*-coumaric acid increased in samples fermented by *P. acidilactici* 5765T, *P. pentosaceus* 4698T and *Lev. brevis* 5354, it was reduced by *Ltp. plantarum* 9567 and consumed by *Ltp. plantarum* 748T (value under the detection limit). Dueñas et al. (2005) reported that trans-p-coumaric and cis-ferulic acids were consumed by *Ltp. plantarum* in cowpeas (*Vigna sinensis* L.), depending on their isomeric form. Likewise, *Lev. brevis*, *Ltp. plantarum* and *P. pentosaceus* were able to metabolise *p*-coumaric and ferulic acids through decarboxylation pathway. In addition, Filannino et al. (2015) demonstrated that *p*-coumaric acid was degraded into *p*-vinyl-phenol or reduced in dihydro-*p*-coumaric acid (phloretic acid) by *Ltp. plantarum* in cherry juice, underlining a strain-specific metabolism. However, the decrease of *p*-coumaric acid in *Ltp. plantarum* 748T fermented sample was not explained by the amount of its reduced metabolite accumulation.

Cinnamoyl ester hydrolases, also known as cinnamoyl esterase, can cause the hydrolysis reaction of hydroxycinnamoyl esters (Esteban-Torres et al., 2015), releasing free acids as substrate for phenolic acid decarboxylases PAD (Landete et al., 2010). In *Ltp. plantarum* 748T fermented sample, *p*-coumaroyl glycolic acid, an ester of *p*-coumaric acid, was not detected. This result suggested an initial hydrolysis by a cinnamoyl esterase enzyme (Esteban-Torres et al., 2015), followed by a decarboxylase of *p*-coumaric acid. Cinnamoyl esterase activity is important in the de-esterification process of dietary fibre in human and ruminal digestion improving the antioxidant, anti-inflammatory and antimicrobial activity of complex dietary compounds (Muñoz et al., 2017).

Chlorogenic acid, also known as 3-caffeoylquinic acid, was significantly decreased in the analysed samples, especially with *P. acidilactici* 5765T, but this compound was not consumed by *Ltp. plantarum* 748T (Sánchez-Maldonado et al., 2011).

Fermented avocado leaves samples were characterised by an increase in the detected amount of rutin isomers, in particular in the presence of *P. acidilactici* 5765T, with respect to the control. Nevertheless, after the fermentation guided by *Ltp. plantarum* 748T rutin isomers a and b were reduced, but isomer c was increased significantly. Quercetin-3-glucoside isomers were characterised by the same behaviour of rutin compounds. Lin et al. (2014) reported that quercetin-3-glucoside, as well as kaempferol, kaempferol-3-glucoside and quercetin, were released from rutin

after fermentation with *Aspergillus awamori* in Litchi pericarp. Likewise, a significant increase of kaempferol was observed only in the sample fermented by *P. pentosaceus* 4695T. Hydrolysis of rutin to kaempferol-3-rutinoside or quercetin-3-glucoside is catalysed by α-rhamnosidases and further hydrolysed by β-glucosidases to free kaempferol or quercetin, respectively (Leonard et al., 2021). However, free quercetin was detected under the limit of determination in all analysed samples.

The enzymatic activity of bacteria can break down vegetable cell walls and release bound phenolics improving their bioavailability and facilitating their extraction (Muñoz et al., 2017; Wang et al., 2021). Flavonoids were the main phenolic compound found in avocado leaves and were highly increased after fermentations with LAB strains. These compounds were represented mostly by quercetin and kaempferol (Lee et al., 2015). In general, the concentration of glucoside flavonoids, such as luteolin-7-O-(2”-O-pentosyl)-hexoside isomer a, quercetin-diglucoside isomer a and b, and quercetin-3-O-arabinosyl-glucoside isomer a, increased if compared to the not-fermented control. On the other hand, luteolin-7-O-(2”-O-pentosyl)-hexoside isomer d and quercetin-3-glucoside isomer b were significantly reduced by *Ltp. plantarum* 748T. As reported by Landete et al. (2015), *Ltp. plantarum* 748T strains transformed food aryl glycosides (phloridzin, esculin, daidzin and salicin) into aglycones, with the exception of quercetin glucoside which remained glycosylated until the end of fermentation process. It is reported that the de-glycosylation is associated with an increase in the antioxidant activity (Landete et al., 2014). Moreover, the decrease in concentration of quercetin glucoside isomer b, but not in isomer a, suggested a glycosyl hydrolase activity dependent on the isomeric form.

In the gut, conjugated glucosides are hydrolysed by the intestinal microbiota to be absorbed into their corresponding aglycone, which show higher activity than their precursor (Filannino et al., 2018). β-glycosidase activity is widespread among LAB and have a significant positive impact on fermented products, improving their flavour and organoleptic characteristics (Michlmayr and Kneifel, 2014). Glycosidase activity of *Ltp. plantarum* has been associated to an improvement of the bioavailability of food phenolic compounds, with an increase in their antioxidant activity (Landete et al., 2014). Although *Ltp. plantarum* 748T decreased total phenolic content of avocado leaves, contrary to *Ltp. plantarum* 9567, it showed a significant decrease of aryl-glucosides, suggesting a higher glycosidase activity than the other strains. These results could be related to the high antioxidant activity found through DPPH and FRAP assays, with respect to the other fermented samples.

A hierarchical clustering heatmap was performed to provide an intuitive visualisation of all the phenolic compounds quantified by HPLC-ESI-TOF-MS in the fermented avocado leaves by the

selected LAB strains (Figure 6.3). The colour scale describes the concentration values of each compound, ranged from a normalised value of 2 (intense red) to -2 (intense blue).

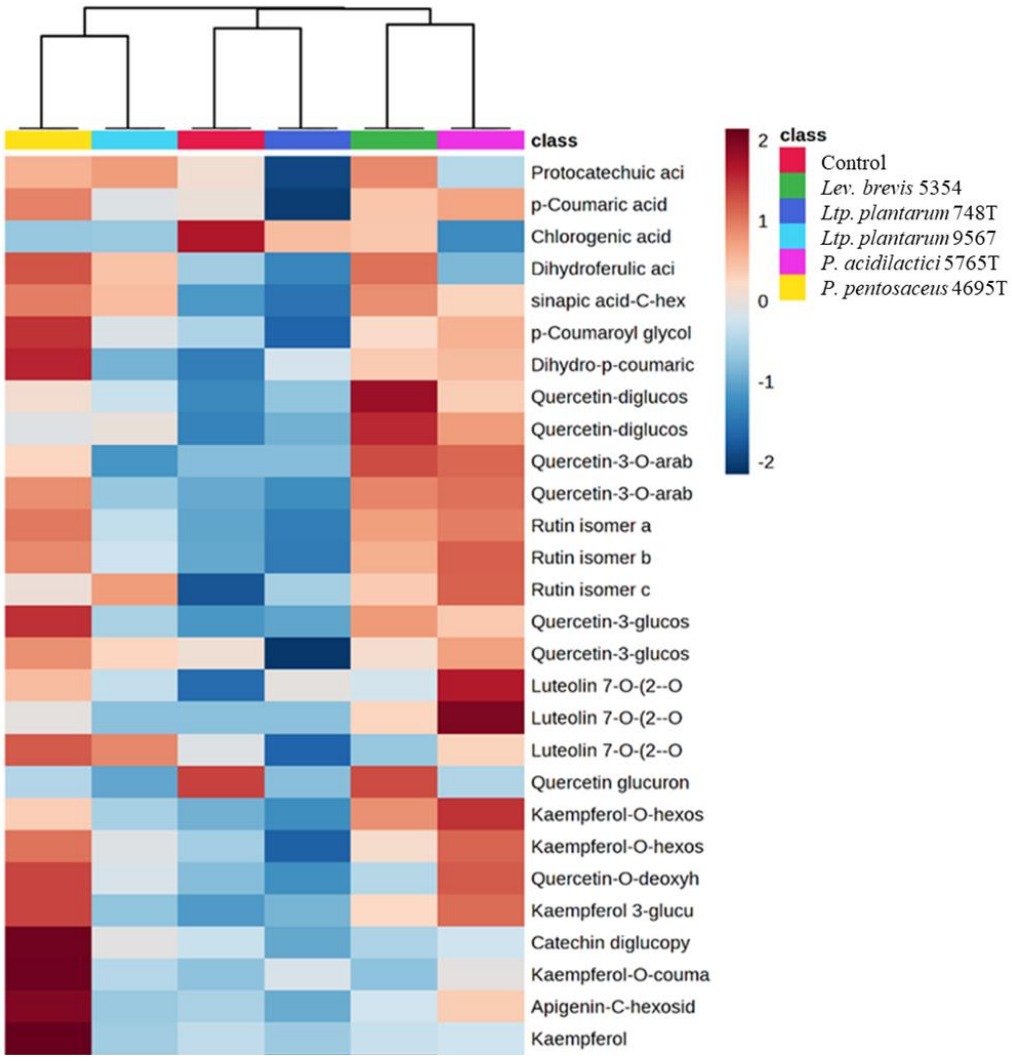


Figure 6.3: Heatmap of fermented avocado leaves samples, clustered through their phenolic compounds content.

The sample fermented by *Ltp. plantarum* 748T was clustered with the control, due to the fact that they were characterised by small differences in the phenolic profile. Moreover, these samples had the lowest total phenolic content and the highest amount of chlorogenic acid. Meanwhile, *Lev. brevis* 5354 and *P. acidilactici* 5765T were grouped together because showed higher content of luteolin and quercetin derivatives. Finally, the cluster composed by *P. pentosaceus* 4695T and *Ltp. plantarum* 9567 seemed to be a heterogeneous group in terms of amounts of single compounds, but with similar profile and proportions between them.

This clustering analysis confirmed that the formation of phenolic compounds depends on the activation of LAB strain-specific metabolic pathway. Moreover, LAB metabolism depends on the capability of strains to tolerate and hydrolyse these compounds.

6.1.4 Conclusions

This part of the PhD thesis allowed to identify the chemical biotransformation induced by the fermentation of avocado leaves by selected LAB strains. A total of 48 polar compounds were identified by HPLC-ESI-TOF-MS, 15 of which have been identified for the first time in this type of vegetal matrix. The obtained results showed that the samples with *Ltp. plantarum* 748T and *P. pentosaceus* 4695T were characterised by the highest antioxidant activity. Moreover, with the exception of *Ltp. plantarum* 748T, fermentations with *P. acidilactici* 5765T, *P. pentosaceus* 4695T, *Ltp. plantarum* 9567 and *Lev. brevis* 5354 led to an increase of the total phenolic content, as demonstrated by HPLC-ESI-TOF-MS.

In conclusion, biotransformation induced by bacterial metabolism modified the phenolic compound profile of avocado leaves in a strain-specific dependent manner, suggesting that LAB led the hydrolysis of compounds by enzymatic activity, such as glycosidases or decarboxylase, and the release of bound phenolics from plant cell walls, thus improving their bioavailability. For these reasons, LAB strains can be used in the exploitation and valorisation of avocado agro-wastes for the production of enriched phenolic extracts.

Data published as:

De Montijo-Prieto; S., Razola-Diaz, M.C., **Barbieri, F.**, Tabanelli, G., Gardini, F., Jiménez-Valera, M., Ruiz-Bravo, A., Verardo, V.*, Gómez-Caravaca, A.M. (2023). Impact of LAB fermentation on phenolic compounds and antioxidant activity of avocado leaf extracts. *Antioxindant*, 12, 298. doi: 10.3390/antiox12020298.

Chapter 7:

General conclusion

7.1 Conclusions

As described in the objectives, this thesis was structured on three main strands of research.

The first concerned the use of autochthonous microbial strains, isolated from spontaneously fermented sausages collected in Mediterranean countries, to be proposed as bio-protective or functional starter cultures. In this perspective, the work started from the isolation of LAB from 15 fermented sausages produced in four European countries (Italy, Slovenia, Spain and Croatia) and from a metagenomic analysis addressed to highlight the biodiversity of their microbiota (Chapter 3.1). As expected, LAB and CNC were the most representative microorganisms in all samples, but significant differences were observed in relation to the characteristics and provenience of these fermented sausages. *Latilactobacillus* was the only genus found in all products, even if LAB belonging to the genus *Companilactobacillus* were found, especially in Spanish samples. These differences were reflected in the first instance in fermented sausages safety characteristics, *i.e.* biogenic amines concentrations. Moreover, also the volatilome, and consequently the peculiar sensory features of traditional products, was dependent on the complexity of the microbiota, which allowed to compare the microbial composition and the final characteristics of fermented sausages. The bacterial isolates were genetically identified allowing the definition of approx. 150 biotypes, mainly belonging to the species *Lat. sakei* followed, albeit in smaller quantity, by *Lat. curvatus*, *Comp. alimentarius*, *Lactiplantibacillus paraplantarum* and *Pediococcus acidilactici* (Chapter 3.2). These identifications did not confirm the great species biodiversity found through the metagenomic analysis and highlighted the high assertiveness and specialisation of *Lat. sakei* for this ecological niche.

After the exclusion of the strains producing biogenic amine or showing antibiotic-resistance, according to EFSA indications, only safe strains (approx. 40) were characterised for their technological properties. In particular, the strain growth performances at different temperatures and in the presence of different NaCl concentrations were considered together with the potential to produce volatile compounds in meat model. In addition, these strains were tested for their inhibiting activity against some pathogens and analysed for the presence of genes related to bacteriocin production (Chapter 3.3). This preliminary screening work was preparatory to the selection of the most interesting strains for possible industrial applications.

In this perspective, in this PhD thesis two examples of applications are proposed in meat products obtained at pilot plant scale. The first concerned the use of two strains chosen for their anti-listerial activity (*Ltp. paraplantarum* and *P. acidilactici*) as bio-protective cultures in fresh sausages (Chapter 4.1) and the second the use of the same strains as starter cultures for the preparation of fermented sausages (Chapter 4.2). The results obtained were encouraging, in

particular for the fermented sausages, in which salamis produced with the two selected strains presented good sensory properties and aroma profile and were characterised by a marked reduction in BA content. In addition, the growth of *List. monocytogenes*, assessed in a challenge test, was strongly inhibited.

Other application trails in this issue are ongoing on pilot plant or industrial productions, also testing other autochthonous strains besides those mentioned. These activities were performed in the frame of the project BioProMedFood (PRIMA – Section 2), of which this PhD thesis is a part. Although the results are not reported in this thesis, as they are not yet finalised, the preliminary data demonstrated a very promising future for the use of these new strains, that could be exploited also at industrial level.

The second objective of this PhD thesis was focused on the use of natural compounds with antimicrobial potential. The attention has been focused on the bioactivity of molecules present in plant derivatives obtained from species characterising the Mediterranean maquis, in particular *Juniperus oxycedrus* and *Rubus fruticosus*. Plant derivatives were obtained with two processes: hydroalcoholic extraction (plant phenolic extract) and hydro-distillation (plant essential oil). After the characterisation of their composition and the evaluation of their MIC *in vitro*, their antimicrobial activity was tested against a strain of *List. monocytogenes* (Chapter 5.1) and *Ent. faecium* (Chapter 5.2). In the latter case the bioactivity was also tested for the inhibition of tyramine production. In both cases, a more in-depth study was conducted to assess the nature of the damage suffered by the microbial cells and their ability to repair the injuries over time by using a flow cytometric protocol. Relevant differences were observed concerning the effects in relation to the species and in relation to the type of derivative, *i.e.*, phenolic extract or essential oil. These *in vitro* studies are fundamental to understand the potentiality of these natural substances and their possible application in food to maximise and optimise their activities.

The last aim of this PhD thesis was the evaluation of the use of fermentation of plant by-products to obtain plant extracts enriched in phenolic compounds. In relation to this issue, research work is still ongoing, in collaboration with Prof. Verardo (Department of Nutrition and Food Sciences, University of Granada) and several perspectives are still open. The first data obtained, however, are encouraging (Chapter 6.1). In fact, an increase in antioxidant potential of avocado (*Persea americana*) leaf extracts, following fermentation by different LAB species (*P. acidilactici*, *P. pentosaceus*, *Leuc. mesenteroides*, *Lev. brevis*, *Ltp. plantarum*), has been demonstrated. Biotransformations induced by bacterial metabolism modified the phenolic compound profile of avocado leaves in a strain-specific dependent manner, suggesting that LAB led to the hydrolysis of

compounds by enzymatic activity, such as glycosidases or decarboxylase, and the release of bound phenolics from plant cell walls, thus improving their bioavailability.

Concluding, the work presented in this PhD thesis permitted to highlight two aspects. The first aspect is the great potential of traditional meat products to serve as a reservoir of microbial biodiversity for the bioprospecting of interesting new strains with industrial importance. The second aspect highlighted is that plant material, especially that of minor, under-exploited matrices, can represent an important reservoir of compounds and extracts obtainable at low-cost, and therefore available even by local producers, to increase the shelf-life and safety of fresh foods from a “green” perspective.

These aspects demonstrate the possibility of applying optimised biotechnological tools that can represent crucial innovative strategies for pursuing production efficiency and promoting the sustainability of the entire production system from a circular economy perspective.

As noted above, the work presented in this PhD thesis is continuing, and future results may provide better evidence of the potential use of the studied strategies, to be proposed also at the industrial level and in matrices other than those considered in this work, such as fresh seafood and ready-to-eat products.

Bibliography

Abdelhamid, A., Jouini, M., Bel Haj Amor, H., Mzoughi, Z., Dridi, M., Ben Said, R., Bouraoui, A. (2018). Phytochemical analysis and evaluation of the antioxidant, anti-inflammatory, and antinociceptive potential of phlorotannin-rich fractions from three Mediterranean brown seaweeds. *Mar. Biotechnol.*, 20, 60-74. doi: 10.1007/s10126-017-9787-z.

Abebe, E., Gugsa, G., Ahmed, M. (2020). Review on major food-borne zoonotic bacterial pathogens. *J. Trop. Med.*, 2020, 4674235. doi: 10.1155/2020/4674235.

Abriouel, H., Valdivia, E., Martínez-Bueno, M., Maqueda, M., Gálvez, A. (2003). A simple method for semi-preparative-scale production and recovery of enterocin AS-48 derived from *Enterococcus faecalis* subsp. *liquefaciens* A-48-32. *J. Microbiol. Methods*, 55 599-605. doi:10.1016/S0167-7012(03)00202-1.

Adamczak, A., Ożarowski, M., Karpiński, T.M. (2020). Antibacterial activity of some flavonoids and organic acids widely distributed in plants. *J. Clin. Med.*, 9, 109. doi: 10.3390/jcm9010109.

Ağagündüz, D., Özge Şahin, T., Ayten, Ş., Yılmaz, B., Güneşliol, B.E., Russo, P., Spano, G., Özogul, F. (2022). Lactic acid bacteria as pro-technological, bioprotective and health-promoting cultures in the dairy food industry. *Food Biosci.*, 47, 101617. doi: 10.1016/j.fbio.2022.101617.

Agrimonti, C., White, J. C., Tonetti, S., Marmiroli, N. (2019). Antimicrobial activity of cellulosic pads amended with emulsions of essential oils of oregano, thyme and cinnamon against microorganisms in minced beef meat. *Int. J. Food Microbiol.*, 305, 108246. doi: 10.1016/J. IJFOODMICRO.2019.108246.

Al-Enazi, N.M., Awaad, A.S., Zain, M.E., Alqasoumi, S.I. (2018). Antimicrobial, antioxidant and anticancer activities of *Laurencia catarinensis*, *Laurencia majuscula* and *Padina pavonica* extracts. *Saudi Pharm. J.*, 26, 44-52. doi: 10.1016/j.jsps.2017.11.001.

Al-Gamal, M.S., Ibrahim, G.A., Sharaf, O.M., Radwan, A.A., Dabiza, N.M., Youssef, A.M., El-Ssayad, M.F. (2019). The protective potential of selected lactic acid bacteria against the most common contaminants in various types of cheese in Egypt. *Heliyon*, 5, e01362. doi: 10.1016/j.heliyon.2019.e01362.

Al_husnan, L.A., Alkahtani, M.D.F. (2016). Impact of Moringa aqueous extract on pathogenic bacteria and fungi *in vitro*. *Ann. Agric. Sci.*, 61, 247-250. doi: 10.1016/j.aoas.2016.06.003.

Albuquerque, T.G., Santos, F., Sanches-Silva, A., Oliveira, M.B., Bento, A.C., Costa, H.S. (2016). Nutritional and phytochemical composition of Annona cherimola Mill. fruits and by-products: potential health benefits. *Food Chem.*, 193, 187-195. doi: 10.1016/j.foodchem.2014.06.044.

Alghazeer, R., Elmansori, A., Sidati, M., Gammoudi, F., Azwai, S., Naas, H., Garbaj, A., Eldaghayes, I. (2017). *In vitro* antibacterial activity of flavonoid extracts of two selected Libyan algae against

multi-drug resistant bacteria isolated from food products. *J. Biosci. Med.*, 5, 26-48. doi: 10.4236/jbm.2017.51003.

Almeida-Santos, A.C., Novais, C., Peixe, L., Freitas, A.R. (2021). *Enterococcus* spp. as a producer and target of bacteriocins: a double-edged sword in the antimicrobial resistance crisis context. *Antibiotics*, 10, 1215. doi: 10.3390/antibiotics10101215.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. (1990). Basic local alignment search tool. *J. Mol. Biol.*, 215, 403-410. doi: 10.1016/S0022-2836(05)80360-2.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, A., Miller, W., Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.*, 25, 3389-3402. doi: 10.1093/nar/25.17.3389.

Álvarez-Martínez, F.J., Barrajón-Catalán, E., Herranz-López, M., Micol, V. (2021). Antibacterial plant compounds, extracts and essential oils: an updated review on their effects and putative mechanisms of action. *Phytomedicine*, 90, 153626. doi: 10.1016/j.phymed.2021.153626.

Ameer, A., Seleshe, S., Kim, B.J., Kang, A.S.N. (2021). Inoculation of *Lactobacillus sakei* on quality traits of dry fermented sausages. *Prev. Nutr. Food Sci.*, 26, 476-484. doi: 10.3746/pnf.2021.26.4.476.

Amorim, A.M., Nardelli, A.E., Chow, F. (2020). Effects of drying processes on antioxidant properties and chemical constituents of four tropical macroalgae suitable as functional bioproducts. *J. Appl. Phycol.*, 32, 1495-1509. doi: 10.1007/s10811-020-02059-7.

Ananou, S., Garriga, M., Jofré, A., Aymerich, T., Gálvez, A., Maqueda, M., Martínez-Bueno, M., Valdivia, E. (2010). Combined effect of enterocin AS-48 and high hydrostatic pressure to control food-borne pathogens inoculated in low acid fermented sausages. *Meat Sci.*, 84, 594–600. doi: 10.1016/j.meatsci.2009.10.017.

Anderegg, J., Fischer, M., Durig, J., Die, A., Lacroix, C., Meile, L. (2020). Detection of biogenic amines and tyramine-producing bacteria in fermented sausages from Switzerland. *J. Food Prot.*, 83, 1512-1519. doi: 10.4315/JFP-19-468.

Andersen, G., Marcinek, P., Sulzinger, N., Schieberle, P., Krautwurst, D. (2019). Food sources and biomolecular targets of tyramine. *Nutr. Rev.*, 77, 107-115. doi: 10.1093/nutrit/nuy036.

Aquilanti, L., Garofalo, C., Osimani, A., Clementi, F. (2016). Ecology of lactic acid bacteria and coagulase negative cocci in fermented dry sausages manufactured in Italy and other Mediterranean countries: an overview. *Int. Food Res. J.*, 23, 429-445.

Arena, M.P., Silvain, A., Normanno, G., Grieco, F., Drider, D., Spano, G., Fiocco, D. (2016). Use of *Lactobacillus plantarum* strains as a bio-control strategy against food-borne pathogenic microorganisms. *Frontiers Microbiol.*, 7, 464. doi: 10.3389/fmicb.2016.00464.

Arioli, S., Montanari, C., Magnani, M., Tabanelli, G., Patrignani, F., Lanciotti, R., Mora, D., Gardini, F. (2019). Modelling of *Listeria monocytogenes* Scott A after a mild heat treatment in the presence of thymol and carvacrol: effects on culturability and viability. *J. Food Eng.*, 240, 73-82. doi: 10.1016/j.jfoodeng.2018.07.014.

Arnoldi, M., Comi, G., Iacumin, L. (2022). Antibiotic resistance and virulence factors in lactobacilli: something to carefully consider. *Food Microbiol.*, 103, 103934. doi: 10.1016/j.fm.2021.103934.

Aryana, K.J., Olson, D.W.A. (2017). 100-Year Review: Yogurt and other cultured dairy products. *J. Dairy Sci.*, 100, 9987-10013 doi: 10.3168/jds.2017-12981.

Austrich-Comas, A., Serra-Castelló, C., Jofré, A., Gou, P., Bover-Cid, S. (2022). Control of *Listeria monocytogenes* in chicken dry-fermented sausages with bioprotective starter culture and high-pressure processing. *Front. Microbiol.*, 13, 983265. doi: 10.3389/fmicb.2022.983265.

Axelsson, L. (2004). Lactic acid bacteria: Classification and physiology. In Salminen, S., von Wright, A., Ouwehand, A. (eds.), *Lactic Acid Bacteria: Microbiology and Functional Aspects*, 3rd edition, Marcel Dekker, New York, USA, pp. 1-66.

Ayala-Zavala, J.F., Vega-Vega, V., Rosas-Domínguez, C., Palafox-Carlos, H., Villa-Rodriguez, J.A., Wasim Siddiqui, Md., Dávila-Aviña, J.E., González-Aguilara, G.A. Agro-industrial potential of exotic fruit byproducts as a source of food additives. *Food Res. Int.*, 44, 1866-1874. doi:10.1016/j.foodres.2011.02.021.

Aymerich, T., Rodríguez, M., Garriga, M., Bover-Cid, S. (2019). Assessment of the bioprotective potential of lactic acid bacteria against listeria monocytogenes on vacuum-packed cold-smoked salmon stored at 8°C. *Food Microbiol.*, 83, 64-70. doi: 10.1016/j.fm.2019.04.011.

Aymerich, T., Jofré, A., Bover-Cid, S. (2022). Enterocin A-based antimicrobial film exerted strong antilisterial activity in sliced dry-cured ham immediately and after 6 months at 8°C. *Food Microbiol.*, 105, 104005. doi: 10.1016/j.fm.2022.104005.

Azmir, J., Zaidul, I.S.M., Rahman, M.M., Sharif, K.M., Mohamed, A., Sahena, F., Jahurul, M.H.A., Ghafoor, K., Norulaini, N.A.N., Omar, A.K.M. (2013). Techniques for extraction of bioactive compounds from plant materials: a review. *J. Food Eng.*, 117, 426-436. doi: 10.1016/j.jfoodeng.2013.01.014.

Bahramian, G., Golestan, L., Khosravi-Darani, K. (2018). Antimicrobial and antioxidant effect of nanoliposomes containing *Zataria multiflora* Boiss essential oil on the rainbow trout fillets during refrigeration. Biointerface Res. Appl. Chem., 8, 3505-3513.

Bajpai, V.K., Baek, K.H., Kang, S.C. (2012). Control of *Salmonella* in foods by using essential oils: a review. *Food Res. Int.*, 45, 722-734. doi: 10.1016/j.foodres.2011.04.052.

Bajpai, V.K., Park, I., Lee, J., Shukla, S., Nile, S.H., Chun, H.S., Khan., I., Oh, S.Y., Lee, H., Huh Y.S., Na, M., Han, Y.K. (2019). Antioxidant and antimicrobial efficacy of a biflavonoid, amentoflavone from Nandina domestica in vitro and in minced chicken meat and apple juice food models. *Food Chem.*, 271, 239-247. doi: 10.1016/j.foodchem.2018.07.159.

Balouiri, M., Sadiki, M., Ibnsouda, S.A. (2016). Methods for in vitro evaluating antimicrobial activity: a review. *J. Pharm. Biomed. Anal.*, 6, 71-79. doi: 10.1016/j.jpha.2015.11.005.

Barak, S., Mudgil, D. (2023). Application of bioactives from herbs and spices for improving the functionality and shelf life of dairy products - a review. *Biointerface Res. Appl. Chem.*, 13, 141. doi: 10.33263/BRIAC132.141.

Barbieri, F., Montanari, C., Gardini, F., Tabanelli, G. (2019). Biogenic amine production by lactic acid bacteria: a review. *Foods*, 8, 17. doi: 10.3390/foods8010017.

Barbieri F., Laghi, L., Gardini, F., Montanari, C., Tabanelli, G. (2020). Metabolism of *Lactobacillus sakei* Chr82 in the presence of different amounts of fermentable sugars. *Foods*, 9, 720. doi:10.3390/foods9060720.

Barbieri, F., Laghi, L., Montanari, C., Lan, Q., Levante, A., Gardini, F., Tabanelli, G. (2022). Insights into the metabolomic diversity of *Latilactobacillus sakei*. *Foods*, 11, 477. doi: 10.3390/foods11030477.

Barbosa, M.S., Todorov, S.D., Ivanova, I., Chobert, J.M., Haertlé, T., Franco, B.D.G.M. (2015). Improving safety of salami by application of bacteriocins produced by an autochthonous *Lactobacillus curvatus* isolate. *Food Microbiol.*, 46, 254-262. doi: 10.1016/j.fm.2014.08.004.

Barcenilla, C., Ducic, M., López, M., Prieto, M., Álvarez-Ordóñez, A. (2022a). Application of lactic acid bacteria for the biopreservation of meat products: a systematic review. *Meat Sci.*, 183, 108661. doi: 10.1016/j.meatsci.2021.108661.

Barcenilla, C., Álvarez-Ordóñez, A., López, M., Alvseike, O., Prieto, M. (2022b). Microbiological safety and shelf-life of low-salt meat products - a review. *Foods*, 11, 2331. doi: 10.3390/foods11152331.

Bargossi, E., Gardini, F., Gatto, V., Montanari, C., Torriani, S., Tabanelli, G. (2015a). The capability of tyramine production and correlation between phenotypic and genetic characteristics of *Enterococcus faecium* and *Enterococcus faecalis* strains. *Front. Microbiol.*, 6, 1371. doi: 10.3389/fmicb.2015. 00259.

Bargossi, E., Tabanelli, G., Montanari, C., Gatto, V., Chinnici, F., Gardini, F., Torriani, S. (2017). Growth, biogenic amine production and tyrDC transcription of *Enterococcus faecalis* in synthetic medium containing defined amino acid concentrations. *J. Appl. Microbiol.*, 122, 1078-1091. doi: 10.1111/jam.13406.

Bargossi, E., Tabanelli, G., Montanari, C., Lanciotti, R., Gatto, V., Gardini, F., et al. (2015b). Tyrosine decarboxylase activity of enterococci grown in media with different nutritional potential: Tyramine and 2-phenylethylamine accumulation and *tyrDC* gene expression. Front. Microbiol. 6, 259. doi: 10.3389/fmicb.2015.00259.

Bassi, D., Puglisi, E., Cocconcelli, P.S. (2015). Comparing natural and selected starter cultures in meat and cheese fermentations. *Curr. Opin. Food Sci.*, 2, 118-122. doi: 10.1016/j.cofs.2015.03.002.

Batiha, G.E.S., Alkazmi, L.M., Wasef, L.G., Beshbishy, A.M., Nadwa, E.H., Rashwan, E.K. (2020). *Syzygium aromaticum* L. (*Myrtaceae*): traditional uses, bioactive chemical constituents, pharmacological and toxicological activities. *Biomolecules*, 10, 202. doi: 10.3390/biom10020202.

Ben Said, L., Gaudreau, H., Dallaire, L., Tessier, M., Fliss, I. (2019). Bioprotective culture: a new generation of food additives for the preservation of food quality and safety. *Ind. Biotechnol.*, 15, 138-147. doi: 10.1089/ind.2019.29175.lbs.

Bensid, A., Ucar, Y., Bendeddouche, B., Özogul, F. (2014). Effect of the icing with thyme, oregano and clove extracts on quality parameters of gutted and beheaded anchovy (*Engraulis encrasicholus*) during chilled storage. *Food Chem.*, 145, 681-686. doi: 10.1016/j.foodchem.2013.08.106.

Beretta, C., Stoessel, F., Baier, U., Hellweg, S. (2013). Quantifying food losses and the potential for reduction in Switzerland. Waste Manag., 33, 764-773. doi: 10.1016/j.wasman.2012.11.007.

Bernardo, P., Patarata, L., Lorenzo, J.M., Fraqueza, M.J. (2021). Nitrate is nitrate: the status quo of using nitrate through vegetable extracts in meat products. *Foods*, 10, 3019. doi: 10.3390/foods10123019.

Bernardini, G., Minetti, M., Polizzotto, G., Biazzo, M., Santucci, A. (2018). Pro-apoptotic activity of French Polynesian *Padina pavonica* extract on human osteosarcoma cells. *Mar. Drugs*, 16, 504. doi: 10.3390/md16120504.

Berni, E. (2015). Molds. In Toldrá, F., Hui, Y.H., Astiasarán, I., Sebranek, J.G., Talon, R. (eds.), *Handbook of Fermented Meat and Poultry*, 2nd edition, John Wiley & Sons Inc, Hoboken, USA, pp. 147-154.

Berthoud, H., Wechsler, D., Irmler, S. (2022). Production of putrescine and cadaverine by *Paucilactobacillus wasatchensis*. *Front. Microbiol.*, 13, 842403. doi: 10.3389/fmicb.2022.842403.

Bhavaniramya, S., Vishnupriya, S., Al-Aboody, M.S., Vijayakumar, R., Baskaran, D. (2019). Role of essential oils in food safety: antimicrobial and antioxidant applications. *GOST*, 2, 49-55. doi: 10.1016/J.GAOST.2019.03.001.

Bhattacharya, D., Nanda, P.K., Pateiro, M., Lorenzo, J.M., Dhar, P., Das, A.K. (2022) Lactic acid bacteria and bacteriocins: novel biotechnological approach for biopreservation of meat and meat products. *Microorganisms*, 10, 2058. doi: 10.3390/microorganisms10102058.

Bin, S., Yi-Zhong, C., John, D.B., Harold, C. (2011). Potential application of spice and herb extracts as natural preservatives in cheese. *J. Med. Food*, 14, 284-290. doi: 10.1089/jmf.2010.0009.

Bintsis, T. (2018). Lactic acid bacteria as starter cultures: an update in their metabolism and genetics. *AIMS Microbiol.*, 4, 665-684. doi: 10.3934/microbiol.2018.4.665.

Biris-Dorhoi, E.S., Michiu, D., Pop, C.R., Rotar, A.M., Tofana, M., Pop, O.L., Socaci, S.A., Farcas, A.C. (2020). Macroalgae - a sustainable source of chemical compounds with biological activities. *Nutrients*, 12, 3085. doi: 10.3390/nu12103085.

Biswas, B., Rogers, K., McLaughlin, F., Daniels, D., Yadav, A. (2013). Antimicrobial activities of leaf extracts of guava (*Psidium guajava* L.) on two Gram-negative and Gram-positive bacteria. *Int. J. Microbiol.*, 2013, 746165. doi: 10.1155/2013/746165.

Biswas, O., Kandasamy, P., Patnaik, S., Lorenzo, J.M., Das, A.K. (2021). Effect of phytochemicals on quality and safety aspects of meat and meat products. *Indian J. Anim. Health.*, 60, 97-108. doi: 10.36062/ijah.2021.spl.02921.

Bolívar, A., Tarlak, F., Costa, J.C.C.P., Cejudo-Gómez, M., Bover-Cid, S., Zurera, G., Pérez-Rodríguez, F. (2021). A new expanded modelling approach for investigating the bioprotective capacity of *Latilactobacillus sakei* CTC494 against *Listeria monocytogenes* in ready-to-eat fish products. Food Res. Int., 147, 110545. doi: 10.1016/j.foodres.2021.110545.

Boskou, G., Salta, F.N., Chrysostomou, S., Mylona, A., Chiou, A., Andrikopoulos, N.K. (2006). Antioxidant capacity and phenolic profile of table olives from the Greek market. *Food Chem.*, 94, 558-564. doi: 10.1016/j.foodchem.2004.12.005.

Boudiba, S., Tamfu, N.A., Berka, B., Hanini, K., Hioun, S., Allaf, K., Boudiba, L., Ceylan, O. (2021). Anti-quorum sensing and antioxidant activity of essential oils extracted from Juniperus species, growing spontaneously in Tebessa Region (East of Algeria). *Nat. Prod. Commun.*, 16, 1-11. doi: 10.1177/1934578X211024039.

Bover-Cid, S., Holzapfel, W.H. (1999). Improved screening procedure for biogenic amine production by lactic acid bacteria. *Int. J. Food Microbiol.*, 53, 33-41. doi: 10.1016/S0168-1605(99)00152-X.

Bover-Cid, S., Serra-Castelló, C., Dalgaard, P., Garriga, M., Jofré, A. (2019). New insights on *Listeria monocytogenes* growth in pressurised cooked ham: a piezo-stimulation effect enhanced by organic acids during storage. *Int. J. Food Microbiol.*, 290, 150-158. doi: 10.1016/j.ijfoodmicro.2018.10.008.

Brahmi, F., Mechri, B., Dabbou, S., Dhibi, M., Hammami, M. (2012). The efficacy of phenolics compounds with different polarities as antioxidants from olive leaves depending on seasonal variations. *Ind. Crops Prod.*, 38, 146-152. doi: 10.1016/j.indcrop.2012.01.023.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods - a review. *Int. J. Food Microbiol.*, 94, 223-253. doi: 10.1016/j.ijfoodmicro.2004.03.022.

Busatta, C., Vidal, R.S., Popiolski, A.S., Mossi, A.J., Dariva, C., Rodrigues, M.R.A., Corazza, F.C., Corazza, M.L., Vladimir Oliveira, J., Cansian, R.L. (2008). Application of *Origanum majorana* L. essential oil as an antimicrobial agent in sausage. *Food Microbiol.*, 25, 207-211. doi: 10.1016/j.fm.2007.07.003.

Cabral, C.C., Conte-Junior, C.A., Silva, J.T., Paschoalin, V.M.F. (2014). *Salmonella* spp. contamination in fresh pork and chicken sausages marketed in Niterói and Rio de Janeiro, Brazil. J. *Verbr. Lebensm.*, 9, 243-249. doi: 10.1007/s00003-014-0901-0.

Čagalj, M., Skroza, D., Tabanelli, G., Özogul, F., Šimat, V. (2021). Maximizing the antioxidant capacity of *Padina pavonica* by choosing the right drying and extraction methods. *Processes*, 9, 587. doi: 10.3390/pr9040587.

Čagalj, M., Skroza, D., Razola-Díaz, M.d.C., Verardo, V., Bassi, D., Frleta, R., Generalić Mekinić, I., Tabanelli, G., Šimat, V. (2022). Variations in the composition, antioxidant and antimicrobial activities of *Cystoseira compressa* during seasonal growth. *Mar. Drugs*, 20, 64. doi: 10.3390/md20010064.

Cai, L., Leng, L., Cao, A., Cheng, X., Li, J. (2018). The effect of chitosan-essential oils complex coating on physicochemical, microbiological, and quality change of grass carp (*Ctenopharyhgodon idella*) fillets. *J. Food Saf.*, 38, 12399. doi: 10.1111/jfs.12399.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J., Holmes, S.P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods*, 13, 581-583. doi: 10.1038/nmeth.3869.

Callon, C., Arliguie, C., Montel, M.C. (2016). Control of Shigatoxin-producing *Escherichia coli* in cheese by dairy bacterial strains. *Food Microbiol.*, 53, 63-70. doi: 10.1016/j.fm.2015.08.009.

Calo, J.R., Crandall, P.G., O'Bryan, C.A., Ricke, S.C. (2015). Essential oils as antimicrobials in food systems - a review. *Food Control*, 54, 111-119. doi: 10.1016/j.foodcont.2014.12.040.

Capanoglu, E., Nemli, E., Tomas-Barberan, F. (2022). Novel approaches in the valorization of agricultural wastes and their applications. *J. Agric. Food Chem.*, 70, 6787-6804. doi: 10.1021/acs.jafc.1c07104.

Caponi, C., Catalano, S., Cioni, P.L., Flamini, G., Morelli, I. (2022). Analysis of the volatile fraction of *Rubus ulmifolius* Schott at two different vegetative stages. *J. Essent. Oil Res.*, 14, 63-65. doi: 10.1080/10412905.2002.9699766.

Capozzi, V., Fragasso, M., Russo, P. (2020). Microbiological safety and the management of microbial resources in artisanal foods and beverages: the need for a transdisciplinary assessment to conciliate actual trends and risks avoidance, *Microorganisms*, 8, 306. doi: 10.3390/microorganisms8020306.

Carballo, J. (2012). The role of fermentation reactions in the generation of flavor and aroma of foods. In Mehta, B.M., Kamal-Eldin, A., Iwanski R.Z (eds.), *Fermentation, Effects on food properties*, CRC Press, Boca Raton, USA, pp. 51-83.

Carballo, J. (2021). Sausages: nutrition, safety, processing and quality improvement. *Foods*, 10, 890. doi: 10.3390/foods10040890.

Cardinali, F., Milanović, V., Osimani, A., Aquilanti, L., Taccari, M., Garofalo C., Polverigiani, S., Clementi, F., Franciosi, E., Tuohy, K., Mercuri, M.L., Altissimi M.S., Haouet, M.N. (2018). Microbial dynamics of model Fabriano-like fermented sausages as affected by starter cultures, nitrates and nitrites. *Int. J. Food Microbiol.*, 278, 61-72. doi: 10.1016/j.ijfoodmicro.2018.04.032.

Carminati, D., Giraffa, G., Quiberoni, A., Binetti, A., Suárez, V., Reinheimer, J. (2010). Advances and trends in starter cultures for dairy fermentations. In Mozzi, F., Raya, R.R., Vignolo, G.M. (eds.), *Biotechnology of lactic acid bacteria: novel applications*, Blackwell Publishing, Hoboken, USA, pp. 177-192.

Carramiñana, J.J., Rota, C., Burillo, J., Herrera, A. (2008). Antibacterial efficiency of Spanish Satureja montana essential oil against *Listeria monocytogenes* among natural flora in minced pork. *J. Food Protect.*, 71, 502-508. doi: 10.4315/0362-028X-71.3.502.

Carvalho, R.J., de Souza, G.T., Honório, V.G., de Sousa, J.P., da Conceição, M.L., Maganani, M., de Souza, E.L. (2015). Comparative inhibitory effects of *Thymus vulgaris* L. essential oil against

Staphylococcus aureus, *Listeria monocytogenes* and mesophilic starter co-culture in cheese-mimicking models. *Food Microbiol.*, 52, 59-65. doi: 10.1016/j.fm.2015.07.003.

Carver, T., Harris, S.R., Berriman, M., Parkhill, J., McQuillan, J.A. (2012). Artemis: an integrated platform for visualization and analysis of high-throughput sequence-based experimental data. *Bioinformatics*, 28, 464-469. doi: 10.1093/bioinformatics/btr703.

Casaburi, A., Nasi, A., Ferrocino, I., Di Monaco, R., Mauriello, G., Villani, F., Ercolini, D. (2011). Spoilage-related activity of *Carnobacterium maltaromaticum* strains in air-stored and vacuum-packed meat. *Appl. Environ. Microbiol.*, 77, 7382-7393. doi: 10.1128/AEM.05304-11.

Casaburi, A., Di Martino, V., Ferranti, P., Picariello, L., Villani, F. (2016). Technological properties and bacteriocins production by *Lactobacillus curvatus* 54M16 and its use as starter culture for fermented sausage manufacture. *Food Control*, 59, 31-45. doi: 10.1016/j.foodcont.2015.05.016.

Cassano, A., Conidi, C., Ruby-Figueroa, R., Castro-Muñoz, R. (2018). Nanofiltration and tight ultrafiltration membranes for the recovery of polyphenols from agro-food by-products. *Int. J. Mol. Sci.*, 19, 351. doi: 10.3390/ijms19020351.

Castellano, P., González, C., Carduza, F., Vignolo, G.M. (2010). Protective action of *Lactobacillus curvatus* CRL705 on vacuum-packaged raw beef. Effect on sensory and structural characteristics. *Meat Sci.*, 85, 394-401. doi: 10.1016/j.meatsci.2010.02.007.

Castellano, P., Aristoy, M.C., Sentandreu, M.A., Vignolo, G.M., Toldrá, F. (2012). *Lactobacillus sakei* CRL1862 improves safety and protein hydrolysis in meat systems. *J. Appl. Microbiol.*, 113, 1407-1416. doi: 10.1111/jam.12005.

Castellano, P., Ibarreche, M.P., Massani, M.B., Fontana, C., Vignolo, G.M. (2017). Strategies for pathogen biocontrol using lactic acid bacteria and their metabolites: a focus on meat ecosystems and industrial environments. *Microorganisms*, 5, 38. doi: 10.3390/microorganisms5030038.

Castro, S.M., Silva, J., Casquete, R., Queirós, R., Saraiva, J.A., Teixeira. P. (2018). Combined effect of pediocin bacHA-6111-2 and high hydrostatic pressure to control *L. innocua* in fermented meat sausage Alheira. *Int. Food Res. J.*, 25, 553-560. doi: hdl.handle.net/10400.14/26477.

Castro-López, C., Bautista-Hernández, I., González-Hernández, M.D., Martínez-Ávila, G.C.G., Rojas, R., Gutiérrez-Díez, A., Medina-Herrera, N., Aguirre-Arzola, V.E. (2019). Polyphenolic profile and antioxidant activity of leaf purified hydroalcoholic extracts from seven Mexican *Persea americana* cultivars. *Molecules*, 24. doi: 10.3390/molecules24010173.

CCAES (Centro de Coordinación de Alertas y Emergencias Sanitarias) (2019). Ministerio de Sanidad, Consumo y Bienestar Social. Informe de fin de seguimiento del brote de listeriosis. https://www.mscbs.gob.es/profesionales/saludPublica/ccayes/alertasActual/listeriosis/docs/Informe_cierre_Listeriosis_20190927.pdf. Accessed 30 November 2022.

CDC (Centers for Disease Control and Prevention) (2017). People at risk - Listeria. https://www.cdc.gov/listeria/risk.html. Accessed 30 November 2022.

Cenci-Goga, B.T., Karama, M., Sechi, P., Iulietto, M.F., Novelli, S., Selvaggini, R., Barbera, S. (2016). Effect of a novel starter culture and specific ripening conditions on microbiological characteristics of nitrate-free dry-cured pork sausages. *Ital. J. Anim. Sci.*, 15, 358-374. doi: 10.1080/1828051X.2016.1204633.

Chaillou, S., Christieans, S., Rivollier, M., Lucquin, I., Champomier-Vergès, M.C., Zagorec, M. (2014). Quantification and efficiency of *Lactobacillus sakei* strain mixtures used as protective cultures in ground beef. *Meat Sci.*, 97, 332-338. doi: 10.1016/j.meatsci.2013.08.009.

Chandrakasan, G., Rodríguez-Hernández, A.I., López-Cuellar, M.R., Palma-Rodríguez, H.M., Chavarría-Hernández, N. (2019). Bacteriocin encapsulation for food and pharmaceutical applications: advances in the past 20 years. *Biotechnol. Lett.*, 41, 453-469. doi: 10.1007/s10529-018-02635-5.

Chemat, F., Rombaut, N., Sicaire, A.G., Meullemiestre, A., Fabiano-Tixier, A.S., Abert-Vian, M. (2017). Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications-a review. *Ultrason. Sonochem.*, 34, 540-560. doi: 10.1016/j.ultsonch.2016.06.035.

Chiboub, O., Ktari, L., Sifaoui, I., López-Arencibia, A., Reyes-Batlle, M., Mejri, M., Valladares, B., Abderrabba, M., Piñero, J.E., Lorenzo-Morales, J. (2017). *In vitro* amoebicidal and antioxidant activities of some Tunisian seaweeds. *Exp. Parasitol.*, 183, 76-80. doi: 10.1016/j.exppara.2017.10.012.

Chikindas, M.L., Weeks, R., Drider, D., Chistyakov, V.A., Dicks, L.M. (2018). Functions and emerging applications of bacteriocins. *Curr. Opin. Biotechnol.*, 49, 23-28. doi: 10.1016/j.copbio.2017.07.011.

Chouhan, S., Sharma, K., Guleria, S. (2017). Antimicrobial activity of some essential oils-present status and future perspectives. *Medicines*, 4, 58. doi: 10.3390/medicines4030058.

Chun, J., Oren, A., Ventosa, A., Christensen, H., Arahal, D.R., Da Costa, M.S., Rooney, A.P., Yi, H., Xu, X.W., De Meyer, S., Trujillo, M.E. (2018). Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. *Int. J. Syst. Evol. Microbiol.*, 68, 461-466. doi: 10.1099/ijsem.0.002516.

Churchward, C.P., Alany, R.G., Snyder, L.A.S. (2018). Alternative antimicrobials: the properties of fatty acids and monoglycerides. *Crit. Rev. Microbiol.*, 44, 561-570. doi: 10.1080/1040841X.2018.1467875.

Cifá, D., Skrt, M., Pittia, P., Di Mattia, C., Poklar Ulrih, N. (2018). Enhanced yield of oleuropein from olive leaves using ultrasound-assisted extraction. *Food Sci. Nutr.*, 6, 1128-1137. doi: 10.1002/fsn3.654.

Cocconcelli, P.S., Fontana, C. (2010). Starter cultures for meat fermentation. In Toldrá, F. (ed.), *Handbook of Meat Processing*, Blackwell Publishing, Ames, USA, pp. 199-218.

Cocconcelli, P.S., Fontana, C. (2014). Bacteria. In Toldrá, F., Hui, Y.H., Astiasarán, I., Sebranek, J.G., Talon, R. (eds.), *Handbook of Fermented Meat and Poultry*, 2nd edition, John Wiley & Sons Inc, Hoboken, USA, pp. 13-17.

Cocolin, L., Dolci, P., Rantsiou, K. (2011). Biodiversity and dynamics of meat fermentations: the contribution of molecular methods for a better comprehension of a complex ecosystem. *Meat Sci.*, 89, 296e302. doi: 10.1016/j.meatsci.2011.04.011.

Coelho, M.C., Silva, C.C.G., Ribeiro, S.C., Dapkevicius, M., Rosa, H.J.D. (2014). Control of Listeria monocytogenes in fresh cheese using protective lactic acid bacteria. *Int. J. Food Microbiol.*, 191, 53-59. doi: 10.1016/j.ijfoodmicro.2014.08.029.

Coelho, M.C., Malcata, F.X., Silva, C.C.G. (2022). Lactic acid bacteria in raw-milk cheeses: from starter cultures to probiotic functions. *Foods*, 11, 2276. doi: 10.3390/foods11152276.

Comi, G., Muzzin, A., Corazzin, M., Iacumin, L. (2020). Lactic acid bacteria: variability due to different pork breeds, breeding systems and fermented sausage production technology. *Foods*, 9, 338. doi: 10.3390/foods9030338.

Comunian, R., Daga, E., Dupré, I., Paba, A., Devirgiliis, C., Piccioni, V., Perozzi, G., Zonenschain, D., Rebecchi, A., Morelli, L., Lorentiis, A.D., Giraffa, G. (2010). Susceptibility to tetracycline and erythromycin of *Lactobacillus paracasei* strains isolated from traditional Italian fermented foods. *Int. J. Food Microbiol.*, 138, 151-156. doi: 10.1016/j.ijfoodmicro.2009.11.018.

Coppola, S., Mauriello, G., Aponte, M., Moschetti, G., Villani, F. (2000). Microbial succession during ripening of Naples-type salami, a southern Italian fermented sausage. *Meat Sci.*, 56, 321-329. doi: 10.1016/s0309-1740(00)00046-2.

Corona, L., Mele, C., Chessa, I., Mulas, M. (2017). Analysis of Sardinian myrtle (*Myrtus communis* L.) germplasm selections by SSR markers. *Acta Hortic.*, 1172, 165-170. doi: 10.17660/ActaHortic.2017.1172.32.

Cosentino, S., Barra, A., Pisano, B., Cabizza, M., Pirisi, F.M., Palmas, F. (2003). Composition and antimicrobial properties of Sardinian Juniperus essential oils against foodborne pathogens and spoilage microorganisms. *J. Food Prot.*, 66, 1288-1291. doi: 10.4315/0362-028x-66.7.1288.

Costa, J.C.C.P., Bover-Cid, S., Bolívar, A., Zurera, G., Pérez-Rodríguez, F. (2019). Modelling the interaction of the sakacin-producing *Lactobacillus sakei* CTC494 and *Listeria monocytogenes* in filleted gilthead sea bream (*Sparus aurata*) under modified atmosphere packaging at isothermal and non-isothermal conditions. *Int. J. Food Microbiol.*, 297, 72-84. doi: 10.1016/j.ijfoodmicro.2019.03.002.

Costa, J.C.C.P., Bolivar, A., Valero, A., Carrasco, E., Zurera, G., Perez-Rodriguez, F. (2020). Evaluation of the effect of *Lactobacillus sakei* strain L115 on *Listeria monocytogenes* at different conditions of temperature by using predictive interaction models. *Food Res. Int.*, 131, 108928. doi: 10.1016/j.foodres.2019.108928.

Cotas, J., Leandro, A., Monteiro, P., Pacheco, D., Figueirinha, A., Gonçalves, A.M.M., Da Silva, G.J., Pereira, L. (2020). Seaweed phenolics: from extraction to applications. *Mar. Drugs*, 18, 384. doi: 10.3390/md18080384.

Coton, M., Lebreton, M., Salas, M.L., Garnier, L., Navarri, M., Pawtowski, A., Le Blay, G., Valence, F., Coton, E., Mounier, J. (2018). Biogenic amine and antibiotic resistance profiles determined for lactic

acid bacteria and a propionibacterium prior to use as antifungal bioprotective cultures. *Int. Dairy J.*, 85, 21-26. doi: 10.1016/j.idairyj.2018.05.001.

Cotter, P.D., Ross, R.P., Hill, C. (2013). Bacteriocins - a viable alternative to antibiotics? *Nat. Rev. Microbiol.*, 11, 95-105. doi: 10.1038/nrmicro2937.

Curiel, J.A., Pinto, D., Marzani, B., Filannino, P., Farris, G.A., Gobbetti, M., Rizzello, C.G. (2015). Lactic acid fermentation as a tool to enhance the antioxidant properties of *Myrtus communis* berries. *Microb. Cell Fact.*, 14, 1-10. doi: 10.1186/s12934-015-0250-4.

Cushnie, T.P.T., Lamb, A.J. (2005). Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Agents*, 26, 343-356. doi: 10.1016/j.ijantimicag.2005.09.002.

da Costa, R.J., Voloski, F.L.S., Mondadori, R.G., Duval, E.H., Fiorentini, Â.M. (2019). Preservation of meat products with bacteriocins produced by lactic acid bacteria isolated from meat. *J. Food Qual.*, 2019, 4726510. doi: 10.1155/2019/4726510.

da Silva, B.D., Bernardes, P.C., Pinheiro, P.F., Fantuzzi, E., Roberto, C.D. (2021). Chemical composition, extraction sources and action mechanisms of essential oils: natural preservative and limitations of use in meat products. *Meat Sci.*, 176, 108463. doi: 10.1016/j.meatsci.2021.108463.

Daliri, F., Aboagye, A.A., Daliri, E.B.M. (2020). Inactivation of foodborne pathogens by lactic acid bacteria. *J. Food Hyg. Saf.*, 35, 419-429. doi: 10.13103/JFHS.2020.35.5.419.

Damania, P., Patel, R., Shaw, R., Kataria, R.P., Wadia, A. (2016). Development of antimicrobial packaging materials for food preservation using bacteriocin from *Lactobacillus casei*. *Microbiol. Res.*, 7, 6622. doi:10.4081/mr.2016.6622.

Das, K., Choudhary, R., Thompson-Witrick, K.A. (2019). Effects of new technology on the current manufacturing process of yogurt-to increase the overall marketability of yogurt. *LWT-Food Sci. Technol.*, 108, 69-80. doi: 10.1016/j.lwt.2019.03.058.

Das, A.K., Nanda, P.K., Madane, P., Biswas, S., Das, A., Zhang, W., Lorenzo, J.M. (2020). A comprehensive review on antioxidant dietary fibre enriched meat-based functional foods. Trends *Food Sci. Technol.*, 99, 323-336. doi: 10.1016/j.tifs.2020.03.010.

Davey, H.M. (2011). Life, death, and in-between: meanings and methods in microbiology. *Appl. Environ. Microbiol.*, 77, 5571-5576. doi: 10.1128/AEM.00744-11.

Davis, N.M., Proctor, D.M., Holmes, S.P., Relman, D.A., Callahan, B.J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome*, 6, 226. doi: 10.1186/s40168-018-0605-2.

Daza, M.V.B, Milani, G., Cortimiglia, C., Pietta, E., Bassi, D., Cocconcelli, P.S. (2022). Genomic insight of *Enterococcus faecium* UC7251, a multi-drug resistance strain from ready-to-eat foods, highlights the risk of antimicrobial resistance in the food chain. *Front. Microbiol.*, 13, 894241. doi: 10.3389/fmicb.2022.894241.

de Andrade, M.L., Rodrigues, R.R., Antongiovanni, N., da Cunha, D.T. (2019). Knowledge and risk perceptions of foodborne disease by consumers and food handlers at restaurants with different food safety profiles. *Food Res. Int.*, 121, 845-853. doi: 10.1016/j.foodres.2019.01.006.

de Castilho, A.N.P., Todorova, S.D., Oliveira, L.L., dos Santos Bersot, L., Nero, L.A. (2020). Inhibition of *Listeria monocytogenes* in fresh sausage by bacteriocinogenic *Lactobacillus curvatus* UFV-NPAC1 and its semi-purified bacteriocin. *LWT-Food Sci. Technol.*, 118, 10857. doi: 10.1016/j.lwt.2019.108757.

de Lira, M.H.P., de Andrade Júnior, F.P., Queiroga Moraes, G.F., da Silva Macena, G., de Oliveira Pereira, F., Oliveira Lima, I. (2020). Antimicrobial activity of geraniol: an integrative review. *J. Essent. Oil Res.*, 32, 187-197. doi: 10.1080/10412905.2020.1745697.

Delcarlo, S.B., Parada, R., Schelegueda, L.I., Vallejo, M., Marguet, E.R., Campos, C.A. (2019). From the isolation of bacteriocinogenic LAB strains to the application for fish paste biopreservation. *LWT-Food Sci. Technol.*, 110, 239–246. doi: 10.1016/j.lwt.2019.04.079.

Dentice Maidana, S., Ficoseco, C.S., Bassi, D., Cocconcelli, P.S., Puglisi, E., Savoy, G., Vignolo, G., Fontana, C. (2020). Biodiversity and technological-functional potential of lactic acid bacteria isolated from spontaneously fermented chia sourdough. *Int. J. Food Microbiol.*, 316, 108425. doi: 10.1016/j.ijfoodmicro.2019.108425.

Dey, T.B., Chakraborty, S., Jain, K.K., Sharma, A., Kuhad, R.C. (2016). Antioxidant phenolics and their microbial production by submerged and solid state fermentation process: a review. *Trends Food Sci, Technol.*, 53, 60-74. doi: 10.1016/j.tifs.2016.04.007.

Di Gioia, D., Mazzola, G., Nikodinoska, I., Aloisio, I., Langerholc, T., Rossi, M., Raimondi, S., Melero, B., Rovira, J. (2016). Lactic acid bacteria as protective cultures in fermented pork meat to prevent *Clostridium* spp. growth. *Int. J. Food Microbiol.*, 235, 53-59. doi: 10.1016/j.ijfoodmicro.2016.06.019.

Diaz de Cerio, E., Gómez-Caravaca, A.M., Verardo, V., Fernández-Gutiérrez, A., Segura-Carretero, A. (2016). Determination of guava (*Psidium guajava* L.) leaf phenolic compounds using HPLC-DAD-QTOF-MS. *J. Funct. Foods*, 22, 376-388. doi: 10.1016/j.jff.2016.01.040.

Diaz de Cerio, E., Aguilera-Saez, L.M., Gómez-Caravaca, A.M., Verardo, V., Fernández-Gutiérrez, A., Fernández, I., Arráez-Román, D. (2018). Characterization of bioactive compounds of *Annona cherimola* L. leaves using a combined approach based on HPLC-ESI-TOF-MS and NMR. *Anal. Bioanal. Chem.*, 410, 3607-3619. doi: 10.1007/s00216-018-1051-5.

Diniz-Silva, H.T., Brandão, L.R., de Sousa Galvão, M., Madruga, M.S., Maciel, J.F., de Souza, E.L., Magnani, M. (2020). Survival of *Lactobacillus acidophilus* LA-5 and *Escherichia coli* O157:H7 in Minas Frescal cheese made with oregano and rosemary essential oils. *Food Microbiol.*, 86, 103348. doi: 10.1016/j.fm.2019.103348.

Directive 2006/52/EC of The European Parliament and of the Council of 5 July 2006 amending Directive 95/2/EC on food additives other than colours and sweeteners and Directive 94/35/EC on sweeteners for use in foodstuffs (2006). *Official Journal of the European Union* L 204/10, 26.7.2006.

Direzione Sanità della Regione Piemonte (2011). Linee guida per l'analisi del rischio nel campo della microbiologia degli alimenti, progetto regionale "*Analisi del rischio microbiologico legato al consumo di alimenti finalizzato alla riduzione dei costi analitici*", approvato con Determinazione della Direzione Sanità della Regione Piemonte n.780 del 18 ottobre 2011.

Dortu, C., Huch, M., Holzapfel, W., Franz, C., Thonart, P. (2008). Anti-listerial activity of bacteriocin-producing *Lactobacillus curvatus* CWBI-B28 and *Lactobacillus sakei* CWBI-B1365 on raw beef and poultry meat. *Lett. Appl. Microbiol.*, 47, 581-586. doi: 10.1111/j.1472-765X.2008.02468.x.

dos Santos Cruxen, C.E., Funck, G.D., Haubert, L., da Silva Dannenberg, G., de Lima Marques, J., Chaves, F.C., da Silva, W.P., Fiorentini, Â.M. (2019). Selection of native bacterial starter culture in the production of fermented meat sausages: application potential, safety aspects, and emerging technologies. *Food Res. Int.*, 122, 371-382. doi: 10.1016/j.foodres.2019.04.018.

Dueñas, M., Fernández, D., Hernández, T., Estrella, I., Muñoz, R. (2005). Bioactive phenolic compounds of cowpeas (*Vigna sinensis* L). Modifications by fermentation with natural microflora and with *Lactobacillus plantarum* ATCC 14917. *J. Sci. Food Agric.*, 85, 297-304. doi: 10.1002/jsfa.1924.

Dueñas, M., Hernández, T., Estrella, I. (2007). Changes in the content of bioactive polyphenolic compounds of lentils by the action of exogenous enzymes. Effect on their antioxidant activity. *Food Chem.*, 101, 90-97. doi: 10.1016/j.foodchem.2005.11.053.

Dussault, D., Vu, K.D., Lacroix, M. (2014). In vitro evaluation of antimicrobial activities of various commercial essential oils, oleoresin and pure compounds against food pathogens and application in ham. *Meat Sci.*, 96, 514-520. doi: 10.1016/j.meatsci.2013.08.015.

Dziedzinski, M., Kobus-Cisowska, J., Szymanowska, D., Stuper-Szablewska, K., Baranowska, M. (2020). Identification of polyphenols from coniferous shoots as natural antioxidants and antimicrobial compounds. *Molecules*, 25, 3527. doi: 10.3390/molecules25153527.

EFSA (2011). Scientific opinion on risk-based control of biogenic amine formation in fermented foods. *EFSA J.*, 9, 2393-2486. doi: 10.2903/j.efsa.2011.2393.

EFSA (2015). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. *EFSA J.*, 13, 3991. doi: 10.2903/j.efsa.2015.3991.

EFSA (2021). The European union one health 2019 zoonoses report. *EFSA J.*, 19, 6406. doi: 10.2903/j.efsa.2021.6406.

EFSA Biohaz (EFSA Panel on Biological Hazards). (2018). Scientific opinion on the *Listeria monocytogenes* contamination of ready-to-eat foods and the risk for human health in the EU. *EFSA J.*, 16, 5134. doi: 10.2903/j. efsa.2018.5134.

EFSA Biohaz (EFSA Panel on Biological Hazards). (2021). Statement on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 13:

suitability of taxonomic units notified to EFSA until September 2020. *EFSA J.*, 19, 6377. doi: 10.2903/j.efsa.2021.6377.

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). (2012). Scientific opinion guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA J.*, 10, 2740. doi: 10.2903/j.efsa.2012.2740.

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). (2017). Guidance on the characterisation of microorganisms used as feed additives or as production organisms. *EFSA J.*, 16, 5206. doi: 10.2903/j.efsa.2018.5206.

Egan, K., Field, D., Rea, M.C., Ross, R.P., Hill, C., Cotter, P.D. (2016). Bacteriocins: novel solutions to age old spore-related problems? *Front. Microbiol.*, 7, 461. doi: 10.3389/fmicb.2016.00461.

Ekrikaya, S., Yilmaz, E., Celik, C., Demirbuga S., Ildiz, N., Demirbas, A., Ocsoy, I. (2021). Investigation of ellagic acid rich-berry extracts directed silver nanoparticles synthesis and their antimicrobial properties with potential mechanisms towards *Enterococcus faecalis* and *Candida albicans*. *J. Biotechnol.*, 341, 155-162. doi: 10.1016/j.jbiotec.2021.09.020.

El Abed, N., Kaabi, B., Smaali, M.I., Chabbouh, M., Habibi, K., Mejri, M., Marzouki, M.N., Ahmed, S.B.H. (2014). Chemical composition, antioxidant and antimicrobial activities of thymus capitata essential oil with its preservative effect against *Listeria monocytogenes* inoculated in minced beef meat. *Evid. Based Complement. Alternat. Med.*, 2014, 152487. doi: 10.1155/2014/152487.

El Adab, S., Hassouna, M. (2016). Proteolysis, lipolysis and sensory characteristics of a Tunisian dry fermented poultry meat sausage with oregano and thyme essential oils. *J. Food Saf.*, 36, 19-32. doi: 10.1111/jfs.12209.

Elias, M., Fraqueza, M.J., Laranjo, M. (2018). Biogenic amines in food: presence and control measures. In Stadnik, j. (ed.), *Biogenic amines (BA): origins, biological importance and human health implications*, Nova Science Publishers, Inc, New York, USA, pp. 129-176.

El-Kholy W., Aamer, R. (2017). Effect of some essential oils on the quality of UF-soft cheese during storage. *Alex. J. Food Sci. Tech.*, 14, 13-28. doi: 10.12816/0038401.

Elshreef, H.S., Mirghani, M.E.S., Sulaiman, S., Jami, M.S. (2021). A review of the effect of UAE optimization parameters on antioxidant activity. *IOP Conf. Ser. Earth Environ. Sci.*, 765, 012109. doi: 10.1088/1755-1315/765/1/012109.

Elsser-Gravesen, D., Elsser-Gravesen, A. (2014). Biopreservatives. *Adv. Biochem. Eng. Biotechnol.*, 143, 29-49. doi: 10.1007/10_2013_234.

Erdoğmuş, S.F., Erişmiş, U.C., Uğuz, C. (2021). Isolation and identification of lactic acid bacteria from fermented meat products and evaluation of their antimicrobial effect. *Czech J. Food Sci.*, 39, 289-296. doi: 10.17221/222/2020-CJFS.

Erkmen, O. (2009). Survival of *Staphylococcus aureus* and aerobic bacteria in sucuks made from starter culture and *Thymbra spicata* during manufacturing and storage. *Food Bioprod. Process.*, 87, 62-67. doi:10.1016/j.fbp.2008.02.006.

Eryiğit, T., Okut, N., Ekici, K., Yildirim, B. (2014). Chemical composition and antibacterial activities of *Juniperus horizontalis* essential oil. *Can. J. Plant Sci.*, 94, 323327. doi: 10.4141/CJPS2013-242.

Esteban-Torres, M., Landete, J.M., Reverón, I., Santamaría, L., de las Rivas, B., Muñoz, R. (2015). A *Lactobacillus plantarum* esterase active on a broad range of phenolic esters. *Appl. Environ. Microbiol.*, 81, 3235-3242. doi: 10.1128/AEM.00323-15.

European Commission. (2004). Regulation (EC) no 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs. *Off. J. Eur. Union*, L139, 1-54.

European Commission (2005). Commission regulation (EC) no 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. *Off. J. Eur. Communities*, L338, 1-26. doi: 10.3969/j.issn.1673-8225.2009.50.026.

Falcão, S., Bacém, I., Igrejas, G., Rodrigues, P.J., Vilas-Boas, M., Amaral, J.S. (2018). Chemical composition and antimicrobial activity of hydrodistilled oil from juniper berries. *Ind. Crops Prod.*, 124, 878-884. doi: 10.1016/j.indcrop.2018.08.069.

Fan, S., Qi, Y., Shi, L., Giovani, M., Zaki, N.A.A., Guo, S., Suleria, H.A.R. (2022). Screening of phenolic compounds in rejected avocado and determination of their antioxidant potential. *Processes*, 10. doi: 10.3390/pr10091747.

FAO (2011). Global food losses and food waste - extent, causes and prevention. Food Agric. Organ., Rome.

FAO (2014). The water-energy-food Nexus: a new approach in support of food security and sustainable agriculture. Food Agric. Organ., Rome.

FAO (2015). Global initiative on food loss and waste reduction - “save food”. Food Agric. Organ., Rome.

FAO (2021). Global Agriculture towards 2050. Food Agric. Organ., Rome.

Faour-Klingbeil, D., Todd, E.C.D. (2002). Prevention and control of foodborne diseases in Middle-East North African countries: review of national control systems. *Int. J. Environ. Res. Public Health*, 17, 70. doi: 10.3390/ijerph17010070.

Favaro, L., Barretto Penna, A.L., Todorov, S.D. (2015). Bacteriocinogenic LAB from cheeses – application in biopreservation? *Trends Food Sci. Technol.*, 41, 37-48. doi: 10.1016/J.TIFS.2014.09.001.

Fayad, S., Nehmé, R., Tannoury, M., Lesellier, E., Pichon, C., Morin, P. (2017). Macroalga *Padina pavonica* water extracts obtained by pressurized liquid extraction and microwave-assisted extraction inhibit hyaluronidase activity as shown by capillary electrophoresis. J. *Chromatogr. A.*, 1497, 19-27. doi: 10.1016/j.chroma.2017.03.033.

Ferlemi, A.V., Lamari, F.N. (2016). Berry leaves: an alternative source of bioactive natural products of nutritional and medicinal value. *Antioxidants*, 5, 17. doi: 10.3390/antiox5020017.

Ferroukhi, R., Nagpal, D., Lopez-Peña, A., Hodges, T., Mohtar, R.H., Daher, B., Mohtar, S., Keulertz, M. (2015). *Renewable energy in the water, energy & food Nexus*. International Renewable Energy Agency (IRENA).

Figueroa, J.G., Borrás-Linares, I., Del Pino-García, R., Curiel, J.A., Lozano-Sánchez, J., Segura-Carretero, A. (2021). Functional ingredient from avocado peel: microwave-assisted extraction, characterization

and potential applications for the food industry. *Food Chem.*, 352. doi: 10.1016/j.foodchem.2021.129300.

Filannino, P., Cardinali, G., Rizzello, C.G., Buchin, S., De Angelis, M., Gobbetti, M., Di Cagno, R. (2014). Metabolic responses of *Lactobacillus plantarum* strains during fermentation and storage of vegetable and fruit juices. *Appl. Environ. Microbiol.*, 80, 2206-2215. doi: 10.1128/AEM.03885-13.

Filannino, P., Bai, Y., Di Cagno, R., Gobbetti, M., Gänzle, M.G. (2015). Metabolism of phenolic compounds by *Lactobacillus* spp. during fermentation of cherry juice and broccoli puree. *Food Microbiol.*, 46, 272-279. doi: 10.1016/j.fm.2014.08.018.

Filannino, P., Di Cagno, R., Gobbetti, M. (2018). Metabolic and functional paths of lactic acid bacteria in plant foods: get out of the labyrinth. *Curr. Opin. Biotechnol.*, 49, 64-72. doi: 10.1016/j.copbio.2017.07.016.

Filannino, P., Tlais, A.Z.A., Morozova, K., Cavoski, I., Scampicchio, M., Gobbetti, M., Di Cagno, R. (2020). Lactic acid fermentation enriches the profile of biogenic fatty acid derivatives of avocado fruit (*Persea americana* Mill.). *Food Chem.*, 317, 1-9. doi: 10.1016/j.foodchem.2020.126384.

Flores, M., Corral, S., Cano-García, L., Salvador, A., Belloch, C. (2015). Yeast strains as potential aroma enhancers in dry fermented sausages. *Int. J. Food Microbiol.*, 212, 16-24. doi: 10.1016/j.ijfoodmicro.2015.02.028.

Flores, M. (2018). Understanding the implications of current health trends on the aroma of wet and dry cured meat products. *Meat Sci.*, 144, 53-61. doi: 10.1016/j.meatsci.2018.04.016.

Fontán; M.C.G, Lorenzo, J.M.; Parada, A.; Franco, I., Carballo, J. (2007a). Microbiological characteristics of "androlla", a Spanish traditional pork sausage. *Food Microbiol.*, 24, 52-58. doi: 10.1016/j.fm.2006.03.007.

Fontán, M.C.G., Lorenzo, J.M., Martínez, S., Franco, I., Carballo, J. (2007b). Microbiological characteristics of Botillo, a Spanish traditional pork sausage. *LWT-Food Sci. Technol.*, 40, 1610-1622. doi: 10.1016/j.lwt.2006.10.007.

Fontana, C., Cocconcelli, P.S., Vignolo, G., Saavedra, L. (2015). Occurrence of antilisterial structural bacteriocins genes in meat borne lactic acid bacteria. *Food Control*, 47, 53-59. doi: 10.1016/j.foodcont.2014.06.021.

Fontana, C., Patrone, V., Lopez, C.M., Morelli, L., Rebecchi, A. (2021). Incidence of tetracycline and erythromycin resistance in meat-associated bacteria: impact of different livestock management strategies. *Microorganisms*, 9, 2111. doi: 10.3390/microorganisms9102111.

Franciosa, L., Alessandria, V., Dolci, P., Rantsiou, K., Cocolin, L. (2018). Sausage fermentation and starter cultures in the era of molecular biology methods. *Int. J. Food Microbiol.*, 279, 26-32. doi: 10.1016/j.ijfoodmicro.2018.04.038.

Franciosa, I., Ferrocino, I., Giordano, M., Mounier, J., Rantsiou, K., Cocolin, L. (2021). Specific metagenomic asset drives the spontaneous fermentation of Italian sausages. *Food Res. Int.*, 144, 110379. doi: 10.1016/j.foodres.2021.110379.

Franciosa, I., Ferrocino, I., Corvaglia, M.R., Giordano, M., Coton, M., Mounier, J., Rantsiou, K., Cocolin, L. (2022). Autochthonous starter culture selection for Salame Piemonte PGI production. *Food Res. Int.*, 162, 112007. doi: 10.1016/j.foodres.2022.112007.

Franz, C.M.A.P., Huch, M., Abriouel, H., Holzapfel, W., Gálvez, A. (2011). Enterococci as probiotics and their implications in food safety. *Int. J. Food Microbiol.*, 151, 125-140. doi: 10.1016/j.ijfoodmicro.2011. 08.014.

Fraqueza, M.J. (2015). Antibiotic resistance of lactic acid bacteria isolated from dry-fermented sausages. *Int. J. Food Microbiol.*, 212, 76-88. doi: 10.1016/j.ijfoodmicro.2015.04.035.

Fraqueza, M.J., Laranjo, M., Elias, M., Patarata, L. (2020). Microbiological hazards associated with salt and nitrite reduction in cured meat products: control strategies based on antimicrobial effect of natural ingredients and protective microbiota. *Curr. Opin. Food Sci.*, 38, 32-39. doi: 10.1016/j.cofs.2020.10.027.

Freiding, S., Gutsche, K.A., Ehrmann, M.A., Vogel, R.F. (2011). Genetic screening of *Lactobacillus sakei* and *Lactobacillus curvatus* strains for their peptidolytic system and amino acid metabolism, and comparison of their volatilomes in a model system. *Syst. Appl. Microbiol.*, 34, 311-320. doi: 10.1016/j.syapm.2010.12.006.

Fretin, M., Chassard, C., Delbès, C., Lavigne, R., Rifa, E., Theil, S., Fernandez, B., Laforce, P., Callon, C. (2020). Robustness and efficacy of an inhibitory consortium against *E. coli* O26:H11 in raw milk cheeses. *Food Control*, 115, 107282. doi: 10.1016/j.foodcont.2020.107282.

FSIS (2012). FSIS compliance guideline: controlling *Listeria monocytogenes* in post-lethality exposed ready-to-eat meat and poultry products. Food Service and Inspection Service, Washington, USA.

Gaio, I., Saggiorato, A.G., Treichel, H., Cichoski, A.J., Astolfi, V., Cardoso, R.I., Toniazzo, G., Valduga, E., Paroul, N., Cansian, R. L. (2015). Antibacterial activity of basil essential oil (*Ocimum basilicum* L.) in Italian-type sausage. *J. Verbrauch. Lebensm.*, 10, 323-329. doi:10.1007/s00003-015-0936-x.

Gajewski, J., Pavlovic, R., Fischer, M., Boles, E., Grininger, M. (2017). Engineering fungal *de novo* fatty acid synthesis for short chain fatty acid production. *Nat. Commun.*, 8, 14650. doi: /10.1038/ncomms14650.

Galanakis, C. (2015). *Food Waste Recovery: Processing Technologies and Industrial Techniques*. Academic Press, Cambridge, USA. ISBN: 978-0-12-800351-0.

Gao, Z., Daliri, E.B.M., Wang, J.U.N., Liu, D., Chen, S., Ye, X., Ding, T. (2019). Inhibitory effect of lactic acid bacteria on foodborne pathogens: a review. *J. Food Prot.*, 82, 441-453. doi: 10.4315/0362-028X.JFP-18-303.

García-Díez, J., Alheiro, J., Pinto, A.L., Soares, L., Falco, V., Fraqueza, M.J., Patarata, L. (2016). Behaviour of food-borne pathogens on dry cured sausage manufactured with herbs and spices essential oils and their sensorial acceptability. *Food Control*, 59, 262-270. doi: 10.1016/j.foodcont.2015.05.027.

García-Díez, J., Saraiva, C. (2021). Use of starter cultures in foods from animal origin to improve their safety. *Int. J. Environ. Res. Public Health*, 18, 2544. doi: 10.3390/ijerph18052544.

García-Rodríguez, Y.M., Torres-Gurrola, G., Meléndez-González, C., Espinosa-García, F.J. (2016). Phenotypic variations in the foliar chemical profile of *Persea americana* Mill. cv. *Hass*. *Chem. Biodivers.*, 13, 1767-1775. doi: 10.1002/cbdv.201600169.

Gardini, F., Özogul, Y., Suzzi, G., Tabanelli, G., Özogul, F. (2016). Technological factors affecting biogenic amine content in foods: a review. *Front. Microbiol.*, 7, 1218. doi: 10.3389/fmicb.2016.01218.

Gatto, V., Tabanelli, G., Montanari, C., Prodomi, V., Bargossi, E., Torriani, S., Gardini, F. (2016). Tyrosine decarboxylase activity of *Enterococcus mundtii*: new insights into phenotypic and genetic aspects. *Microb. Biotechnol.*, 9, 801-813. doi: 10.1111/1751-7915.12402.

Ghabbour, N., Lamzira, Z., Thonart, P., Cidalia, P., Markaoui, M., Asehraou, A. (2011). Selection of oleuropein-degrading lactic acid bacteria strains isolated from fermenting moroccan green olives. *Grasas y Aceites*, 62, 84-89. doi: 10.3989/gya.055510.

Giarratana, F., Muscolino, D., Ragonese, C., Beninati, C., Sciarrone, D., Ziino, G., Mondello, L., Giuffrida, A., Panebianco, A. (2016). Antimicrobial activity of combined thyme and rosemary essential oils against *Listeria monocytogenes* in Italian mortadella packaged in modified atmosphere: thyme and rosemary EOs *vs L. monocytogenes*. *J. Essen. Oil Res.*, 28, 467-474. doi: 10.1080/10412905.2016.1165744.

Gizaw, Z. (2019). Public health risks related to food safety issues in the food market: a systematic literature review. *Environ. Health Prev. Med.*, 24, 68. doi: 10.1186/s12199-019-0825-5.

Geeraerts, W., Pothakos, V., De Vuyst, L., Leroy, F. (2018). Variability within the dominant microbiota of sliced cooked poultry products at expiration date in the Belgian retail. *Food Microbiol.*, 73, 209-215. doi: 10.1016/j.fm.2018.01.019.

Generalić Mekinić, I., Gotovac, M., Skroza, D., Ljubenkov, I., Burčul, F., Katalinić, V. (2014). Effect of the extraction solvent on the oleuropein content and antioxidant properties of olive leaf (cv. Oblica, Lastovka and Levantinka) extracts. *Croat. J. Food Sci. Technol.*, 6, 8-15.

Generalić Mekinić, I., Skroza, D., Šimat, V., Hamed, I., Čagalj, M., Perković, Z.P. (2019). Phenolic content of brown algae (Pheophyceae) species: extraction, identification, and quantification. *Biomolecules*, 9, 244. doi: 10.3390/biom9060244.

Generalić Mekinić, I., Čagalj, M., Tabanelli, G., Montanari, C., Barbieri, F., Skroza, D., Šimat, V. (2021). Seasonal changes in essential oil constituents of *Cystoseira compressa*: first report. *Molecules*, 26, 6649. doi: 10.3390/molecules26216649.

Gil-Chávez, G.J., Villa, J.A., Ayala-Zavala, J.F., Heredia, J.B., Sepulveda, D., Yahia, E.M., González-Aguilar, G.A. (2013). Technologies for extraction and production of bioactive compounds to be used as nutraceuticals and food ingredients: an overview. *Compr. Rev. Food Sci. Food Saf.*, 12, 5-23. doi: 10.1111/1541-4337.12005.

Giraffa, G. (2003). Functionality of enterococci in dairy products. *Int. J. Food Microbiol.*, 88, 215-222. doi: 10.1016/S0168-1605(03)00183-1.

Gomez, J.S., Parada, R.B., Vallejo, M., Marguet, E.R., Bellomio, A., Perotti, N., de Carvalho, K.G. (2021). Assessment of the bioprotective potential of lactic acid bacteria against *Listeria monocytogenes* in ground beef. *Arch. Microbiol.*, 203, 1427-1437. doi: 10.1007/s00203-020-02118-0.

Gottardi, D., Bukvicki, D., Prasad, S., Tyagi, A.K. (2016). Beneficial effects of spices in food preservation and safety. *Front. Microbiol.*, 7, 1394. doi: 10.3389/fmicb.2016.01394.

Gouveia, A.R., Alves, M., de Almeida, J.M.M.M., Monteiro-Silva, F., González-Aguilar, G., Silva, J.A., Saraiva, C. (2017). The antimicrobial effect of essential oils against *Listeria monocytogenes* in sous vide cook-chill beef during storage. *J. Food Process. Preserv.*, 41, e13066. doi: 10.1111/jfpp.13066.

Graham, K., Stack, H., Rea, R. (2020). Safety, beneficial and technological properties of enterococci for use in functional food applications - a review. *Crit. Rev. Food Sci. Nutr.*, 60, 3836-3861. doi: 10.1080/10408398.2019.1709800.

Gudej, J., Tomczyk, M. (2004). Determination of flavonoids, tannins and ellagic acid in leaves from Rubus L. species. *Arch. Pharm. Res.*, 27, 1114-1119. doi: 10.1007/BF02975114.

Gutiérrez-Fernández, J., García-Armesto, M.R., Álvarez-Alonso, R., del Valle, P., de Arriaga, D., Rúa, J. (2013). Antimicrobial activity of binary combinations of natural and synthetic phenolic antioxidants against *Enterococcus faecalis*. *J. Dairy Sci.*, 96, 4912-4920. doi: 10.3168/jds.2013-6643.

Hammami, R., Fliss, I., Corsetti, A. (2019). Application of protective cultures and bacteriocins for food biopreservation. *Front. Microbiol.*, 10, 1561. doi: 10.3389/fmicb.2019.01561.

Hampikyan, H., Ugur, M. (2007). The effect of nisin on *L. monocytogenes* in Turkish fermented sausages (sucuks). Meat Sci., 76, 327-332. doi: 10.1016/j.meatsci.2006.11.014.

Han, J., Kong, T., Wang, Q., Jiang, J., Zhou, Q., Li, P., Zhu, B., Gu, Q. (2022). Regulation of microbial metabolism on the formation of characteristic flavor and quality formation in the traditional fish sauce during fermentation: a review. *Crit. Rev. Food Sci. Nutr.*, 7, 1-20. doi: 10.1080/10408398.2022.2047884.

Hao, R., Roy, K., Pan, J., Shah, B.R., Mraz, J. (2021). Critical review on the use of essential oils against spoilage in chilled stored fish: a quantitative meta-analyses. *Trends Food Sci. Technol.*, 111, 175-190. doi: 10.1016/j.tifs.2021.02.054.

Hassoun, A., Coban, O.E. (2017). Essential oils for antimicrobial and antioxidant applications in fish and other seafood products. *Trends Food Sci. Technol.*, 68, 26-36. doi: 10.1016/j.tifs.2017.07.016.

He, R., Chen, W., Chen, H., Zhong, Q., Zhang, H., Zhang, M., Zhang, Chen, W. (2022). Antibacterial mechanism of linalool against *L. monocytogenes*, a metabolomic study. *Food Control*, 132, 108533. doi: 10.1016/j.foodcont.2021.108533.

Helkar, P.B., Sahoo, A.K., Patil, N.J. (2016). Review: food industry by-products used as a functional food ingredients. *Int. J. Waste Resour.*, 6, 1000248. doi: 10.4172/2252-5211.1000248.

Helmuth, I.G., Espenhain, L., Ethelberg, S., Jensen, T., Kjeldgaard, J., Litrup, E., Schjørring, S., Müller, L. (2019). An outbreak of monophasic *Salmonella* Typhimurium associated with raw pork sausage and

other pork products, Denmark 2018-19. *Epidemiol. Infect.*, 147, e315. doi: 10.1017/S0950268819002073.

Hlila, M.B., Hichri, A.O., Mahjoub, M.A., Mighri, Z., Mastouri, M. (2017). Antioxidant and antimicrobial activities of *Padina pavonica* and *Enteromorpha* sp. from the Tunisian Mediterranean coast. *J. Coast. Life Med.*, 336-342. doi: 10.12980/jclm.5.2017J7-107.

Hoff, H. (2011). *Understanding the Nexus. Background paper for the Bonn2011 conference: the water, energy and food security Nexus*. Stockholm, Sweden: Stockholm Environment Institute (SEI).

Holck, A., Heir, E., Johannessen, T.C., Axelsson, L. (2015). Northern European products. In Toldrá, F., Hui, Y.H., Astiasarán, I., Sebranek, J.G., Talon, R. (eds.), *Handbook of Fermented Meat and Poultry*, 2nd edition, John Wiley & Sons Inc, Hoboken, USA, pp. 313-320.

Holck, A., Axelsson, L., McLeod, A., Rode, T.M., Heir, E. (2017). Health and safety considerations of fermented sausages. *J. Food Qual.*, 2017, 9753894. doi: 10.1155/2017/9753894.

Hollenbeck, B.L., Rice, L.B. (2012). Intrinsic and acquired resistance mechanisms in enterococcus. *Virulence*, 3, 421-569. doi: 10.4161/viru.21282.

Hu, K., Jin, G.J., Mei, W.C., Li, T., Tao, Y.S. (2018). Increase of medium-chain fatty acid ethyl ester content in mixed *H. uvarum/S. cerevisiae* fermentation leads to wine fruity aroma enhancement. *Food Chem.*, 239, 495-501. doi: 0.1016/j.foodchem.2017.06.151.

Hu, T., Subbiah, V., Wu, H., BK, A., Rauf, A., Alhumaydhi, F.A., Suleria, H.A.R. (2021). Determination and characterization of phenolic compounds from Australia-grown sweet cherries (*Prunus avium* L.) and their potential antioxidant properties. *ACS Omega*, 6, 34687-34699. doi: 10.1021/acsomega.1c05112.

Huang, Z., Liu, X.C., Jia, S.L., Luo, Y.K. (2017). Antimicrobial effects of cinnamon bark oil on microbial composition and quality of grass carp (*Ctenopharyngodon idellus*) fillets during chilled storage. *Food Control*, 82, 316-324. doi: 10.1016/j.foodcont.2017.07.017.

Huang, Z., Liu, X.C., Jia, S.L., Zhang, L.T., Luo, Y.K. (2018). The effect of essential oils on microbial composition and quality of grass carp (*Ctenopharyngodon idellus*) fillets during chilled storage. *Int. J. Food Microbiol.*, 266, 52-59. doi: 10.1016/j.ijfoodmicro.2017.11.003.

Hungerford, J.M. (2010). Scombroid poisoning: a review. *Toxicon*, 56, 231-243. doi: 10.1016/S0963-9969(96)00066-X.

Hungerford, J.M. (2021). Histamine and scombrotoxins. *Toxicon*, 201, 115-126. doi: 10.1016/j.toxicon.2021.08.013.

Hyldgaard, M., Mygind, T., Meyer, R.L. (2012). Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. *Front. Microbiol.*, 3, 1-24. Doi: 10.3389/fmicb.2012.00012.

Iacumin, L., Cappellari, G., Pellegrini, M., Basso, M., Comi, G. (2021). 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. *Front Microbiol.*, 12, 796655 doi: 10.3389/fmicb.2021.796655.

Ioanna, F., Quaglia, N.C., Storelli, M.M., Castiglia, D., Goffredo, E., Storelli, A., de Rosa, M., Normanno, G., Jambrenghi, C., Dambrosio, A. (2018). Survival of *Escherichia coli* O157:H7 during the manufacture and ripening of Cacioricotta goat cheese. *Food Microbiol.*, 70, 200-205. doi: 10.1016/j.fm.2017.10.005.

Irakli, M., Chatzopoulou, P., Ekateriniadou, L. (2018). Optimization of ultrasound-assisted extraction of phenolic compounds: oleuropein, phenolic acids, phenolic alcohols and flavonoids from olive leaves and evaluation of its antioxidant activities. *Ind. Crops Prod.*, 124, 382–388. doi: 10.1016/j.indcrop.2018.07.070.

Ismail, G.A., Gheda, S.F., Abo-Shady, A.M., Abdel-Karim, O.H. (2020). *In vitro* potential activity of some seaweeds as antioxidants and inhibitors of diabetic enzymes. *Food Sci. Technol.*, 40, 681-691. doi: 10.1590/fst.15619.

ISO (2017a). ISO 11290-1:2017. Microbiology of the food chain - horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp. Part 1: detection method. Geneva: International Organization for Standardization.

ISO (2017b). ISO 6579-1: 2017. Microbiology of the food chain - horizontal method for the detection, enumeration and serotyping of *Salmonella*. Part 1: detection of *Salmonella* spp. method. Geneva: International Organization for Standardization.

Jácome, S., Fonseca, S., Pinheiro, R., Todorov, S. D., Noronha, L., Silva, J., Gomesd, A., Pintadod, M., Moraisd, A.M.M.B., Teixeirad, P., Vaz-Velho, M. (2014). Effect of lactic acid bacteria on quality and safety of ready-to-eat sliced cured/smoked meat products. *Chem. Eng. Trans.*, 38, 403-408. doi: 10.3303/CET1438068.

Jahromi, S.G. (2019). Extraction techniques of phenolic compounds from plants. In Chowdhary, V., Alooparampil, S., Pandya, R.V., Tank, J.G. (eds.), *Plant Physiological Aspects of Phenolic Compounds Basic*, IntechOpen, London, UK, pp. 1-18.

Janßen, D., Eisenbach, L., Ehrmann, M.A., Vogel, R.F. (2018). Assertiveness of *Lactobacillus sakei* and *Lactobacillus curvatus* in a fermented sausage model. *Int. J. Food Microbiol.*, 285, 188-197. doi: 10.1016/j.ijfoodmicro.2018.04.030.

Jimenez, P., Garcia, P., Quitral, V., Vasquez, K., Parra-Ruiz, C., Reyes-Farias, M., Garcia-Diaz, D.F., Robert, P., Encina, C., Soto-Covasich, J. (2021). Pulp, leaf, peel and seed of avocado fruit: a review of bioactive compounds and healthy benefits. *Food Rev. Int.*, 37, 619-655. doi: 10.1080/87559129.2020.1717520.

Jobim, M.L., Santos, R.C.V., dos Santos Alves, C.F., Oliveira, R.M., Mostardeiro, C.P., Sagrillo, M.R., de Souza Filho, O.C., Garcia, L.F.M., Manica-Cattani, M.F., Ribeiro, E.E., da Cruz, I.B.M. (2014). Antimicrobial activity of Amazon *Astrocaryum aculeatum* extracts and its association to oxidative metabolism. *Microbiol. Res.*, 169, 314-323. doi: 10.1016/j.micres.2013.06.006.

Jofré, A., Aymerich, T., Garriga, M. (2009). Improvement of the food safety of low acid fermented sausages by enterocins A and B and high pressure. *Food Control*, 20, 179-184. doi: 10.1016/j.foodcont.2008.04.001.

Jones, R.J., Wiklund, E., Zagorec, M., Tagg, J.R. (2010). Evaluation of stored lamb bio-preserved using a three-strain cocktail of *Lactobacillus sakei*. *Meat Sci.*, 86, 955-959. doi: 10.1016/j.meatsci.2010.07.023.

Jurášková, D., Ribeiro, S.C., Silva, C.C. (2022). Exopolysaccharides produced by lactic acid bacteria: from biosynthesis to health-promoting properties. *Foods*, 11, 156. doi: 10.3390/foods11020156.

Kareem, R.A., Razavi, S.H. (2020). Plantaricin bacteriocins: as safe alternative antimicrobial peptides in food preservation - a review. *J. Food Saf.*, 40, e12735. doi: 10.1111/jfs.12735.

Katikou, P., Ambrosiadis, I., Georgantelis, D., Koidis, P., Georgakis, A.S. (2005). Effect of *Lactobacillus*-protective cultures with bacteriocin-like inhibitory substances producing ability on microbiological, chemical and sensory changes during storage of refrigerated vacuum-packaged sliced beef. J. *Appl. Microbiol.*, 99, 1303-1313. doi: 10.1111/j.1365-2672.2005.02739.x.

Khaleque, M.A., Keya, C.A., Hasan, K.N., Hoque, M.M., Inatsu, Y., Bari, M.L. (2016). Use of cloves and cinnamon essential oil to inactivate *Listeria monocytogenes* in ground beef at freezing and refrigeration temperatures. *LWT-Food Sci. Technol.*, 74, 219-223. doi: 10.1016/j.lwt.2016.07.042.

Khan, H., Flint, S., Yu, P.L. (2010). Enterocins in food preservation. *Int. J. Food Microbiol.*, 141, 1-10. doi: 10.1016/j.ijfoodmicro.2010.03.005.

Khoddami, A., Wilkes, M.A., Roberts, T.H. (2013). Techniques for analysis of plant phenolic compounds. *Molecules*, 18, 2328-2375. doi: 10.3390/molecules18022328.

Khorshidian, N., Yousefi, M., Khanniri, E., Mortazavian, A.M. (2018). Potential application of essential oils as antimicrobial preservatives in cheese. *Innov. Food Sci. Emerg. Technol.*, 45, 62-72. doi: 10.1016/j.ifset.2017.09.020.

Khorshidian, N., Khanniri, E., Mohammadi, M., Mortazavian, A.M., Yousefi, M. (2021). Antibacterial activity of pediocin and pediocin-producing bacteria against *Listeria monocytogenes* in meat products. *Front Microbiol.*, 12, 709959 doi: 10.3389/fmicb.2021.709959.

Kim, J.Y., Jung, C.K., Kim, D.H., Kim, S.B. (2012a). Properties of edible biofilm manufactured from yellowfin tuna (*Thunnus albacares*) skin gelatin. *Korean J. Chem. Eng.*, 29, 786-791. doi: 10.1007/s11814-011-0246-7.

Kim, O.S., Cho, Y.J., Lee, K., Yoon, S.H., Kim, M., Na, H., Park, S.C., Jeon, Y.S., Lee, J.H., Yi, H., Won, S., Chun, J. (2012b). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int. J. Syst. Evol. Microbiol.*, 62, 716-721. doi: 10.1099/ijs.0.038075-0.

Kose, S., Ocak, E. (2020). Determination of antioxidant and antimicrobial activity of Herby cheese. *J. Food Process. Preserv.*, 44, e14841. doi: 10.1111/jfpp.14841.

Kranl, K., Schlesier, K., Bitsch, R., Hermann, H., Rohe, M., Böhm, V. (2005). Comparing antioxidative food additives and secondary plant products-use of different assays. *Food Chem.*, 93, 171-175. doi: 10.1016/j.foodchem.2004.11.012.

Krzepiłko, A., Prażak, R., Święciło, A. (2021). Chemical composition, antioxidant and antimicrobial activity of raspberry, blackberry and raspberry-blackberry hybrid leaf buds. *Molecules*, 26, 327. doi: 10.3390/molecules26020327.

Kumar, P., Chatli, M.K., Verma, A.K., Mehta, N., Malav, O.P., Kumar, D., Sharma, N. (2015). Quality, functionality, and shelf life of fermented meat and meat products: a review. *Crit. Rev. Food Sci. Nutr.* 2015, 57, 2844–2856. doi: 10.1080/10408398.2015.1074533.

Ladero, V., Fernández, M., Calles-Enríquez, M., Sánchez-Llana, E., Cañedo, E., Martin, M.C., Alvarez, M.A. (2012). Is the production of the biogenic amines tyramine and putrescine a species-level trait in enterococci? *Food Microbiol.*, 30, 132-138. doi: 10.1016/j.fm.2011.12.016.

Ladha, G., Jeevaratnam, K. (2020). Characterization of purified antimicrobial peptide produced by *Pediococcus pentosaceus* LJR1, and its application in preservation of white leg shrimp. *World J. Microbiol. Biotechnol.*, 36, 72. doi: 10.1007/s11274-020-02847-w.

Lahiri, D., Nag, M., Dutta, B., Sarkar, T., Pati, S., Basu, D., Kari, Z.A., Wei, L.S., Smaoui, S., Goh, K.W., Ray, R.R. (2022). Bacteriocin: a natural approach for food safety and food security. *Front. Bioeng. Biotechnol.*, 10, 1005918. doi: 10.3389/fbioe.2022.1005918.

Lamuela-Raventós, R.M. (2017). Folin-Ciocalteu method for the measurement of total phenolic content and antioxidant capacity. In Apak, R., Capanoglu, E., Shahidi, F. (eds.), *Measurement of Antioxidant Activity & Capacity*, John Wiley & Sons Inc, Hoboken, USA, pp. 107-115.

Landete, J.M., Pardo, I., Ferrer, S. (2008). Regulation of *hdc* expression and HDC activity by enological factors in lactic acid bacteria. *J. Appl. Microbiol.*, 105, 1544.1551. doi: 10.1111/j.1365-2672.2008.03865.x.

Landete, J.M., Rodríguez, H., Curiel, J.A., De Las Rivas, B., Mancheño, J.M., Muñoz, R. (2010). Gene cloning, expression, and characterization of phenolic acid decarboxylase from *Lactobacillus brevis* RM84. J. *Ind. Microbiol. Biotechnol.*, 37, 617-624. doi: 10.1007/s10295-010-0709-6.

Landete, J.M., Curiel, J.A., Rodríguez, H., de las Rivas, B., Muñoz, R. (2014). Aryl glycosidases from *Lactobacillus plantarum* increase antioxidant activity of phenolic compounds. *J. Funct. Foods*, 7, 322-329. doi: 10.1016/j.jff.2014.01.028.

Landete, J.M., Hernández, T., Robredo, S., Dueñas, M., De Las Rivas, B., Estrella, I., Muñoz, R. (2015). Effect of soaking and fermentation on content of phenolic compounds of soybean (*Glycine max* cv. Merit) and mung beans (*Vigna radiata* [L] Wilczek). *Int. J. Food Sci. Nutr.*, 66, 203-209. doi: 10.3109/09637486.2014.986068.

Laranjo, M., Elias, M., Fraqueza, M.J. (2017). The use of starter cultures in traditional meat products. *J. Food Qual.*, 2017, 9546026. doi: 10.1155/2017/9546026.

Laranjo, M., Potes, M.E., Elias, M. (2019) Role of starter cultures on the safety of fermented meat products. *Front. Microbiol.*, 10, 853. doi: 10.3389/fmicb.2019.00853.

Laulund, S., Wind, A., Derkx, P.M.F., Zuliani, V. (2017). Regulatory and safety requirements for food cultures. *Microorganisms*, 5, 28. doi:10.3390/microorganisms5020028.

Lee, N.K., Jeong, J.H., Oh, J., Kim, Y., Ha, Y.S., Jeong, Y.S. (2015). Conversion of flavonols kaempferol and quercetin in mulberry (*Morus Alba* L.) leaf using plant-fermenting *Lactobacillus plantarum*. *J. Food Biochem.*, 39, 765-770. doi: 10.1111/jfbc.12176.

Leonard, W., Zhang, P., Ying, D., Adhikari, B., Fang, Z. (20219. Fermentation transforms the phenolic profiles and bioactivities of plant-based foods. *Biotechnol. Adv.*, 49, 107763. doi: 10.1016/j.biotechadv.2021.107763.

Leroy, F., Geyzen, A., Janssens, M., De Vuyst, L., Scholliers, P. (2013). Meat fermentation at the crossroads of innovation and tradition: a historical outlook. *Trends Food Sci. Technol.*, 31, 130-137. doi: 10.1016/j.tifs.2013.03.008.

Leroy, S., Lebert, I., Talon, R. (2014). Microorganisms in traditional fermented meats dry-fermented sausages and ripened meats: an overview. In Toldrá, F., Hui, Y.H., Astiasarán, I., Sebranek, J.G., Talon, R. (eds.), *Handbook of Fermented Meat and Poultry*, 2nd edition, John Wiley & Sons Inc, Hoboken, USA, pp. 99-106.

Leroy, F., Scholliers, P., Amilien, V. (2015). Elements of innovation and tradition in meat fermentation: conflicts and synergies. *Int. J. Food Sci.*, 212, 2-8. doi: 10.1016/j.ijfoodmicro.2014.11.016.

Lešić, T., Vahčić, N., Kos, I., Zadravec, M., Sinčić Pulić, B., Bogdanović, T., Petričević, S., Listeš, E., Škrivanko, M., Pleadin, J. (2020). Characterization of traditional Croatian household-produced dry-fermented sausages. *Foods*, 9, 990. doi: 10.3390/foods9080990.

Li, P., Luo, H., Kong, B., Liu, Q., Chen, C. (2016). Formation of red myoglobin derivatives and inhibition of spoilage bacteria in raw meat batters by lactic acid bacteria and Staphylococcus xylosus. *LWT-Food Sci. Technol.*, 68, 251-257. doi: 10.1016/j.lwt.2015.12.035.

Li, H., Wang, P., Lan, R., Luo, L., Cao, X., Wang, Y., Wang, Y., Li, H., Zhang, L., Ji, S., Ye, C. (2018). Risk factors and level of *Listeria monocytogenes* contamination of raw pork in retail markets in China. *Front. Microbiol.*, 9, 1090. doi: 10.3389/fmicb.2018.01090.

Li, J., Yang, X., Shi, G., Chang, J., Liu, Z., Zeng, M. (2019). Cooperation of lactic acid bacteria regulated by the AI-2/LuxS system involve in the biopreservation of refrigerated shrimp. *Food Res. Int.*, 120, 679-687. doi: 10.1016/j.foodres.2018.11.025.

Li, B., Lu, S. (2020). The Importance of amine-degrading enzymes on the biogenic amine degradation in fermented foods: a review. *Process Biochem.*, 99, 331-339. doi: 10.1016/j.procbio.2020.09.012.

Lin, S., Zhu, Q., Wen, L., Yang, B., Jiang, G., Gao, H., Chen, F., Jiang, Y. (2014). Production of quercetin, kaempferol and their glycosidic derivatives from the aqueous-organic extracted residue of litchi pericarp with *Aspergillus awamori*. *Food Chem.*, 145, 220-227. doi: 10.1016/j.foodchem.2013.08.048.

Linares, M.B., Garrido, M.D., Martins, C., Patarata, L. (2013). Efficacies of garlic and *L. sakei* in wine-based marinades for controlling *Listeria monocytogenes* and *Salmonella* spp. in *Chouriço de Vinho*, a dry sausage made from wine-marinated pork. *J. Food Sci.*, 78, M719-M724. doi: 10.1111/1750-3841.12094.

Ling, A.L.M., Yasir, S., Matanjun, P., Abu Bakar, M.F. (2015). Effect of different drying techniques on the phytochemical content and antioxidant activity of *Kappaphycus alvarezii*. *J. Appl. Phycol.*, 27, 1717-1723. doi:10.1007/s10811-014-0467-3.

Linton, M., Connolly, M., Houston, L., Patterson, M.F. (2014). The control of *Clostridium botulinum* during extended storage of pressure-treated, cooked chicken. *Food Control*, 37, 104-108. doi: 10.1016/j.foodcont.2013.09.042.

Liu, Y., McKeever, L.C., Malik, N.S.A. (2017). Assessment of the antimicrobial activity of olive leaf extract against foodborne bacterial pathogens. *Front. Microbiol.*, 8, 113. doi: 10.3389/fmicb.2017.00113.

Loessner, M., Guenther, S., Steffan, S., Scherer, S. (2003). A pediocin-producing *Lactobacillus plantarum* strain inhibits *Listeria monocytogenes* in a multispecies cheese surface microbial ripening consortium. *Appl. Environ. Microbiol.*, 69, 1854-1857. doi: 10.1128/AEM.69.3.1854-1857.2003.

López-Cobo, A., Gómez-Caravaca, A.M.; Pasini, F., Caboni, M.F., Segura-Carretero, A., Fernández-Gutiérrez, A. (2016). HPLC-DAD-ESI-QTOF-MS and HPLC-FLD-MS as valuable tools for the determination of phenolic and other polar compounds in the edible part and by-products of avocado. *LWT - Food Sci. Technol.*, 73, 505-513. doi: 10.1016/j.lwt.2016.06.049.

Lorenzo, J.M., Munekata, P.E.S., Domínguez, R. (2017). Role of autochthonous starter cultures in the reduction of biogenic amines in traditional meat products. *Curr. Opin. Food Sci.*, 14, 61-65. doi: 10.1016/j.cofs.2017.01.009.

Lu, S., Ji, H., Wang, Q., Li, B., Li, K., Xu, C., Jiang, C. (2015). The effects of starter cultures and plant extracts on the biogenic amine accumulation in traditional Chinese smoked horsemeat sausages. *Food Control*, 50, 869-875. doi: 10.1016/j.foodcont.2014.08.015.

Macwana, S.J., Muriana, P.M. (2012). A 'bacteriocin PCR array' for identification of bacteriocin-related structural genes in lactic acid bacteria. *J. Microbiol. Methods*, 88, 197-204. doi: 10.1016/j.mimet.2011.11.008.

Mah, J.H., Hwang, H.J. (2009). Inhibition of biogenic amine for mation in a salted and fermented anchovy by *Staphylococcus xylosus* as a protective culture. *Food Control*, 20, 796-801. doi: 10.1016/j.foodcont.2008.10.005.

Mahgoub, S., Ramadan, M.F., El-Zahar, K. (2013). Cold pressed *Nigella sativa* oil inhibits the growth of foodborne pathogens and improves the quality of Domiati cheese. *J. Food Saf.*, 33, 470-480. doi: 10.1111/jfs.12078.

Mahgoub, S.A., El-Mekkawy, R.M., Abd El-Hack, M.E., El-Ghareeb, W.R., Suliman, G.M., Alowaimer, A.N., Swelum, A.A. (2019). Inactivation of *Listeria monocytogenes* in ready-to-eat smoked turkey

meat by combination with packaging atmosphere, oregano essential oil and cold temperature. *AMB Express.*, 9, 54. doi: 10.1186/s13568-019-0775-8.

Mannino, A.M., Vaglica, V., Oddo, E. (2017). Interspecific variation in total phenolic content in temperate brown algae. *J. Biol. Res.*, 90, 26-29. doi: 10.4081/jbr.2017.6578.

Mannino, G., Gentile, C., Porcu, A., Agliassa, C., Caradonna, F., Bertea, C.M. (2020). Chemical profile and biological activity of cherimoya (*Annona cherimola* Mill.) and atemoya (*Annona atemoya*) leaves. *Molecules*, 25, 2612. doi: 10.3390/molecules25112612.

Marcobal, A., de Las Rivas, B., Landete, J.M., Tabera, L., Muñoz, R. (2012). Tyramine and phenylethylamine biosynthesis by food bacteria. *Crit. Rev. Food Sci. Nutr.*, 52, 448-467. doi: 10.1080/10408398.2010.500545.

Marcos, B., Aymerich, T., Garriga, M., Arnau, J. (2013). Active packaging containing nisin and high pressure processing as post-processing listericidal treatments for convenience fermented sausages. Food Control, 30, 325-330. doi: 10.1016/j.foodcont.2012.07.019.

Martin, N.H., Trmčić, A., Hsieh, T.H., Boor, K.J., Wiedmann, M. (2016). The evolving role of coliforms as indicators of unhygienic processing conditions in dairy foods. *Frontiers Microbiol.*, 7, 1549. doi: 10.3389/fmicb.2016.01549.

Martín-García, B., Pimentel-Moral, S., Gómez-Caravaca, A.M., Arráez-Román, D., Segura-Carretero, A. (2020). Box-Behnken experimental design for a green extraction method of phenolic compounds from olive leaves. *Ind. Crops Prod.*, 154, 112741. doi: 10.1016/j.indcrop.2020.112741.

Martín-García, B., Aznar-Ramos, M.J., Verardo, V., Gómez-Caravaca, A.M. (2022a). Development of an effective sonotrode based extraction technique for the recovery of phenolic compounds with antioxidant activities in cherimoya leaves. *Plants*, 11, 2034. doi: 10.3390/Plants11152034.

Martín-García, B., De Montijo-Prieto, S., Jiménez-Valera, M., Carrasco-Pancorbo, A., Ruiz-Bravo, A., Verardo, V., Gómez-Caravaca, A.M. (2022b). Comparative extraction of phenolic compounds from olive leaves using a sonotrode and an ultrasonic bath and the evaluation of both antioxidant and antimicrobial activity. *Antioxidants*, 11, 558. doi: 10.3390/antiox11030558.

Martín-Platero, A.M., Valdivia, E., Maqueda, M., Martínez-Bueno, M. (2007). Fast, convenient, and economical method for isolating genomic DNA from lactic acid bacteria using a modification of the protein "salting-out" procedure. *Anal. Biochem.*, 366, 102-104. doi: 10.1016/j.ab.2007.03.010.

Martins, S., Mussatto, S.I., Martínez-Avila, G., Montañez-Saenz, J., Aguilar, C.N., Teixeira, J.A. (2011). Bioactive phenolic compounds: production and extraction by solid-state fermentation. A review. *Biotechnol. Adv.*, 29, 365-373. doi: 10.1016/j.biotechadv.2011.01.008.

Masyita, A., Sari, R.M., Astuti, A.D., Yasir, B., Rumata, N.R., Emran, T.B., Nainu, F., Simal-Gandara, J. (2022). Terpenes and terpenoids as main bioactive compounds of essential oils, their roles in human health and potential application as natural food preservatives. *Food Chem.*, 13, 100217. doi: 10.1016/j.fochx.2022.100217.

Mathur, H., Beresford, T.P., Cotter, P.D. (2020). Health benefits of lactic acid bacteria (LAB) fermentates. *Nutrients*, 12, 1679. doi: 10.3390/nu12061679.

McCabe-Sellers, B., Staggs, C.G., Bogle, M.L. (2006). Tyramine in foods and monoamine oxidase inhibitor drugs: a crossroad where medicine, nutrition, pharmacy, and food industry converge. *J. Food Comp. Anal.*, 19, S58-S65. doi: 10.1016/j.jfca.2005.12.008.

McLeod, A., Mosleth, E.F., Rud, I., Dos Santos, F.B., Snipen, L., Liland, K.H., Axelsson, L. (2017). Effects of glucose availability in *Lactobacillus sakei*; metabolic change and regulation of the proteome and transcriptome. *PLoS ONE*, 12, e0187542. doi: 10.1371/journal.pone.0187542.

Medina-Torres, N., Ayora-Talavera, T., Espinosa-Andrews, H., Sánchez-Contreras, A., Pacheco, N. (2017). Ultrasound assisted extraction for the recovery of phenolic compounds from vegetable sources. *Agronomy*, 7, 47. doi: 10.3390/agronomy7030047.

Medini, H., Manongiu, B., Aicha, N., Chekir-Ghedira, L., Harzalla-Skhiri, F., Khouja, M.L. (2013). Chemical and antibacterial polymorphism of *Juniperus oxycedrus* ssp. *oxycedrus* and *Juniperus oxycedrus* ssp. *macrocarpa* (*Cupressaceae*) leaf essential oils from Tunisia. *J. Chem.*, 2013, 389252. doi: 10.1155/2013/389252.

Meehan, T.D., Hurlbert, A.H., Gratton, C. (2010). Bird communities in future bioenergy landscapes of the Upper Midwest. *Proc. Natl. Acad. Sci. U.S.A.*, 107, 18533-18538. doi: 10.1073/pnas.1008475107.

Meenu, M., Padhan, B., Patel, M., Patel, R., Xu, B. (2023). Antibacterial activity of essential oils from different parts of plants against *Salmonella* and *Listeria* spp. *Food Chem.*, 404, 134723. doi: 10.1016/j.foodchem.2022.134723.

Mehdizadeh, T., Narimani, R., Mojaddar Langroodi, A., Moghaddas Kia, E., Neyriz-Naghadehi, M. (2018). Antimicrobial effects of *Zataria multiflora* essential oil and *Lactobacillus acidophilus* on *Escherichia coli* O157 stability in the Iranian probiotic white-brined cheese. *J. Food Saf.*, 38, e12476. doi: 10.1111/jfs.12476.

Mei, J., Ma, X., Xie, J. (2019). Review on natural preservatives for extending fish shelf life. *Foods*, 8, 490. doi: 10.3390/foods8100490.

Meloni, D. (2015). Presence of *Listeria monocytogenes* in Mediterranean-style dry fermented sausages. *Foods*, 4, 34-50. doi: 10.3390/foods4010034.

Menezes, N.M.C., Martins, W.F., Longhi, D.A., de Aragão, G.M.F. (2018). Modeling the effect of oregano essential oil on shelf-life extension of vacuum-packed cooked sliced ham. *Meat Sci.*, 139:113-119. doi: 10.1016/j.meatsci.2018.01.017.

Menon, A.N., Padmakumari, K.P. (2005). Studies on essential oil composition of cultivars of black pepper (*Piper nigrum* L.)-V. *J. Essent. Oil Res.*, 17, 153-155. doi: 10.1080/10412905.2005.9698862.

Meral, R., Ceylan, Z., & Kose, S. (2019). Limitation of microbial spoilage of rainbow trout fillets using characterized thyme oil antibacterial nanoemulsions. *J. Food Saf.*, 39, e12644. doi: 10.1111/jfs.12644.

Mi, J.C., Howard, L.R., Prior, R.L., Clark, J.R. (2004). Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry. *J. Sci. Food Agric.*, 84, 1771-1782. doi: 10.1002/jsfa.1885.

Miceli, N., Marino, A., Köroğlu, A., Cacciola, F., Dugo, P., Mondello, L., Taviano, M.F. (2020). Comparative study of the phenolic profile, antioxidant and antimicrobial activities of leaf extracts of five *Juniperus* L. (*Cupressaceae*) taxa growing in Turkey. *Nat. Prod. Res.*, 34, 1636-1641. doi: 10.1080/14786419.2018.1523162.

Michlmayr, H., Kneifel, W. (2014). β-glucosidase activities of lactic acid bacteria: mechanisms, impact on fermented food and human health. *FEMS Microbiol. Lett.*, 352, 1-10. doi: 10.1111/1574-6968.12348.

Minetti, M., Bernardini, G., Biazzo, M., Gutierrez, G., Geminiani, M., Petrucci, T., Santucci, A. (2019). *Padina pavonica* extract promotes in vitro differentiation and functionality of human primary osteoblasts. *Mar. Drugs*, 17, 473. doi: 10.3390/md17080473.

Mohamed, F.A., Salama, H.H., El-Sayed, S.M., El-Sayed, H.S., Zahran, H.A. (2018). Utilization of natural antimicrobial and antioxidant of *Moringa oleifera* leaves extract in manufacture of cream cheese. *J. Biol. Sci.*, 18, 92-106. doi: 10.3923/jbs.2018.92.106.

Mohamed Ahmed, I.A., Al-Juhaimi, F., Adisa, A.R., Adiamo, O.Q., Babiker, E.E., Osman, M.A., Gassem, M.A., Ghafoor, K., Alqah, H.A.S., Elkareem, M.A. (2020). Optimization of ultrasound-assisted extraction of phenolic compounds and antioxidant activity from Argel (*Solenostemma argel* Hayne) leaves using response surface methodology (RSM). *J. Food Sci. Technol.*, 57, 3071-3080. doi: 10.1007/s13197-020-04340-6.

Monger, X.C., Gilbert, A.A., Saucier, L., Vincent, A.T. (2021). Antibiotic resistance: from pig to meat. *Antibiotics*, 10, 1209. doi: 10.3390/antibiotics10101209.

Montanari, C., Bargossi, E., Gardini, A., Lanciotti, R., Magnani, R., Gardini, F., Tabanelli, G. (2016). Correlation between volatile profiles of Italian fermented sausages and their size and starter culture. *Food Chem.*, 192, 736-744. doi: 10.1016/j.foodchem.2015.07.062.

Montanari, C., Gatto, V., Torriani, S. Barbieri, F., Bargossi, E., Lanciotti, R., Grazia, L., Magnani, R., Tabanellia, G., Gardini, F. (2018a). Effects of the diameter on physico-chemical, microbiological and volatile profile in dry fermented sausages produced with two different starter cultures. *Food Biosci.*, 22, 9-18. doi: 10.1016/j.fbio.2017.12.013.

Montanari, C., Barbieri, F., Magnani, M., Grazia, L., Gardini, F., Tabanelli, G. (2018b) Phenotypic diversity of *Lactobacillus sakei* strains. *Front. Microbiol.*, 9, 2003. doi: 10.3389/fmicb.2018.02003.

Montanari, C., Barbieri, F., Gardini, F., Tabanelli, G. (2021a). Competition between starter cultures and wild microbial population in sausage fermentation: a case study regarding a typical Italian salami (*Ventricina*). *Foods*, 10, 2138. doi: 10.3390/foods10092138.

Montanari, C., Tabanelli, G., Barbieri, F., Mora, D., Duncan, R., Gardini, F., Arioli, S. (2021b). *Listeria monocytogenes* sensitivity to antimicrobial treatments depends on cell origin. *Sci. Rep.*, 11, 21263. doi: 10.1038/s41598-021-00767-9.

Moon, H., Kim, N.H., Kim, S.H., Kim, Y., Ryu, J.H., Rhee, M.S. (2017). Teriyaki sauce with carvacrol or thymol effectively controls *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhimurium*, and indigenous flora in marinated beef and marinade. *Meat Sci.*, 129, 147-152. doi: 10.1016/j.meatsci.2017.03.001.

Mora-Sand, A., Ram, A., Castillo-Henr, L., Lopretti-Correa, M., Vega-Baudrit, J.R. (2021). *Persea americana* agro-industrial waste biorefinery for sustainable high-value-added products. *Polymers*, 13, 1727. doi: 10.3390/polym13111727.

Mrid, R.B., Bouchmaa, N., Bouargalne, Y., Ramdan, B., Karrouchi, K., Kabach, I., El Karbane, M., Idir, A., Zyad, A., Nhiri1, M. (2019). Phytochemical characterization, antioxidant and in vitro cytotoxic activity evaluation of *Juniperus oxycedrus* subsp. *oxycedrus* needles and berries. *Molecules*, 24, 502. doi:10.3390/molecules24030502.

Mulas, M., Mulas, G. (2005). Cultivar selection from rosemary (*Rosmarinus officinalis* L.) spontaneous populations in the mediterranean area. *Acta Hortic.*, 676, 127-133. doi: 10.17660/ActaHortic.2005.676.15.

Muñoz, R., de las Rivas, B., López de Felipe, F., Reverón, I., Santamaría, L., Esteban-Torres, M., Curiel, J.A., Rodríguez, H., Landete, J.M. (2017). Biotransformation of phenolics by *Lactobacillus plantarum* in fermented foods. In Frias, J., Martinez-Villaluenga, C., Peñas, E. (eds.), *Fermented Foods in Health and Disease Prevention*, Academic Press, Cambridge, USA, pp. 63-83.

Mytle, N., Anderson, G.L., Doyle, M.P., Smith, M.A. (2006). Antimicrobial activity of clove (*Syzgium aromaticum*) oil in inhibiting *Listeria monocytogenes* on chicken frankfurters. *Food Control*, 17, 102-107. doi: 10.1016/j.foodcont.2004.09.008.

Nakane, R., Iwashina, T. (2015). Flavonol glycosides from the leaves of *Allium macrostemon*. *Nat. Prod. Commun.*, 10, 1381-1382. doi: 10.1177/1934578X1501000817.

Najar, B., Pistelli, L., Buhagiar, J. (2020). Volatilomic analyses of Tuscan *Juniperus oxycedrus* L. and *in vitro* cytotoxic effect of its essential oils on human cell lines. *J. Essent. Oil-Bear. Plants*, 23, 756-771. doi: 10.1080/0972060X.2020.1823891.

Nami, Y., Bakhshayesh, R.V., Jalaly, H.M., Lotfi, H., Eslami, S., Hejazi, M.A. (2019). Probiotic properties of *Enterococcus* isolated from artisanal dairy products. *Front Microbiol.*, 10, 300. doi: 10.3389/fmicb.2019.00300.

Nathaniel, O.B., Selina, A.S., John, K.M., Mercy, B., Sylvester, A.A., Michael, B.M. (2015). Phytoconstituents, antimicrobial and antioxidant properties of the leaves of *Persea americana* Mill cultivated in Ghana. *J. Med. Plants Res.*, 9, 933-939. doi: 10.5897/jmpr2015.5902.

Neri, D., Antoci, S., Iannetti, L., Ciorba, A.B., D'Aurelio, R., Del Matto, I., Di Leonardo, M., Giovannini, A., Prencipe, V.A., Pomilio, F., Santarellia, G.A., Miglioratia, G. (2019). EU and US control

measures on *Listeria monocytogenes* and *Salmonella* spp. in certain ready-to-eat meat products: an equivalence study. *Food Control*, 96, 98-103. Doi: 10.1016/j.foodcont.2018.09.001.

Ni, Z.J., Wang, X., Shen, Y., Thakur, K., Han, J., Zhang, J.G., Hu, F., Wei, Z.J. (2021). Recent updates on the chemistry, bioactivities, mode of action, and industrial applications of plant essential oils. *Trends Food Sci. Technol.*, 110, 78-89. doi: 10.1016/j.tifs.2021.01.070.

Nieto, G. (2020). A review on applications and uses of thymus in the food industry. *Plants*, 9, 1-29. doi: 10.3390/plants9080961.

Niinivaara, F.P. (1955). About influence of bacterial pure cultures on the ripening and color formation of the dry sausage (in German). *Acta Agral. Fenn.*, 85.

Niinivaara, F.P. (1964). Bacterial pure cultures in the manufacture of fermented sausages. *Food Technol.*, 18, 25-31.

NIST (2011). NIST/NIH/EPA Mass Spectral Library, Standard Reference Database 1, NIST 11. Standard Reference Data Program. Gaithersburg, MD, USA: National Institute of Standards and Technology.

Nogueira, J.O.E., Campolina, G.A., Batista, L.R., Alves, E., Caetano, A.R.S., Brandão, R.M., Nelson, D.L., das Graças Cardoso, M. (2021). Mechanism of action of various terpenes and phenylpropanoids against *Escherichia coli* and *Staphylococcus aureus*. *FEMS Microbiol. Lett.*, 368, fnab052. doi: 10.1093/femsle/fnab052.

Ojha, K.S., Kerry, J.P., Duffy, G., Beresford, T., Tiwari, B.K. (2015). Technological advances for enhancing quality and safety of fermented meat products. *Trends Food Sci Technol.*, 44, 105-116. doi: 10.1016/j.tifs.2015.03.010.

Oliveira, M., Ferreira, V., Magalhães, R., Paula Teixeira, P. (2018). Biocontrol strategies for Mediterranean-style fermented sausages. *Food Res. Int.*, 103, 438-449. doi: 10.1016/j.foodres.2017.10.048.

Omer M., Álvarez-Ordoñez, A., Prieto, M., Skjerve, E., Asehun, T., Alvseike, O.A. (2018). A systematic review of bacterial foodborne outbreaks related to red meat and meat products. *Foodborne Pathog. Dis.*, 15, 598-611. doi: 10.1089/fpd.2017.2393.

Omonijo, F.A., Ni, L.J., Gong, J., Wang, Q., Lahaye, L., Yang, C.B. (2018). Essential oils as alternatives to antibiotics in swine production. *Anim. Nutr.*, 4, 126-136. doi: 10.1016/j.aninu.2017.09.001.

Ordóñez, J.A., Hierro, E.M., Bruna, L.M., Hoz, L. (1999). Changes in the components of dry-fermented sausages during ripening. *Crit. Rev. Food Sci. Nutr.*, 39, 329-367. doi: 10.1080/10408699991279204.

Orhan, N., Orhan, I.E., Ergun, F. (2011). Insights into cholinesterase inhibitory and antioxidant activities of five Juniperus species. *Food Chem. Toxicol.*, 49, 2305-2312. doi: 10.1016/j.fct.2011.06.031.

Orihuel, A., Bonacina, J., Vildoza, M.J., Bru, E., Vignolo, G., Saavedra, L., Fadda, S. (2018). Biocontrol of *Listeria monocytogenes* in a meat model using a combination of a bacteriocinogenic strain with curing additives. *Food Res. Int.*, 107, 289-296. doi: 10.1016/j.foodres.2018.02.043.

Ortiz, S., López, V., Garriga, M., Martínez-Suárez, J.V. (2014). Antilisterial effect of two bioprotective cultures in a model system of Iberian chorizo fermentation. *Int. J. Food Sci. Technol.*, 49, 753-758. doi: 10.1111/ijfs.12362.

Oszmiański, J., Wojdyło, A., Nowicka, P., Teleszko, M., Cebulak, T., Wolanin, M. (2015). Determination of phenolic compounds and antioxidant activity in leaves from wild *Rubus* L. species. *Molecules*, 20, 4951-4966; 10.3390/molecules20034951.

Oulahal, N., Degraeve, P. (2022). Phenolic-rich plant extracts with antimicrobial activity: an alternative to food preservatives and biocides? *Front. Microbiol.*, 12, 753518. doi: 10.3389/fmicb.2021.753518.

Özogul, Y., Yuvka, I., Ucar, Y., Durmus, M., Kosker, A. R., Oz, M., Özogul, F. (2017). Evaluation of effects of nanoemulsion based on herb essential oils (rosemary, laurel, thyme and sage) on sensory, chemical and microbiological quality of rainbow trout (*Oncorhynchus mykiss*) fillets during ice storage. *LWT-Food Sci. Technol.*, 75, 677-684. doi: 10.1016/j.lwt.2016.10.009.

Öztürk, F., Gündüz, H., & Sürengil, G. (2021). The effects of essential oils on inactivation of *Listeria monocytogenes* in rainbow trout cooked with sous-vide. *J. Food Process. Preserv.*, 45, e15878. doi: 10.1111/jfpp.15878.

Palavecino Prpich, N.Z., Camprubí, G.E., Cayré, M.E., Castro, M.P. (2021). Indigenous microbiota to leverage traditional dry sausage production. *Int. J. Food Microbiol.*, 2021, 15. doi: 10.1155/2021/6696856.

Park, S.J., Nam, Y.H., Rodriguez, I., Park, J.H., Kwak, H.J., Oh, Y., Oh, M., Park, M.S., Lee, K.W., Lee, J.S., Kim, D.H., Park, Y.H., Moon, I.S., Choung, S.Y., Jeong, K.W., Hong, B.N., Kang, T.H., Kim, S.H. (2019). Chemical constituents of leaves of *Persea americana* (avocado) and their protective effects against neomycin-induced hair cell damage. *Rev. Bras. Farmacogn.*, 29, 739-743. doi: 10.1016/j.bjp.2019.08.004.

Pasini, F., Soglia, F., Petracci, M., Caboni, M.F., Marziali, S., Montanari, C., Gardini, F., Grazia, L., Tabanelli, G. (2018). Effect of fermentation with different lactic acid bacteria starter cultures on biogenic amine content and ripening patterns in dry fermented sausages. *Nutrients*, 10, 1497. doi: 10.3390/nu10101497.

Paulo E.S. Munekata, Chaves-Lopez, C., Fernandez-Lopez, J., Viuda-Martos, M., Sayas-Barbera, M.S., Perez-Alvarez, J.A., Lorenzo, J.M. (2022). Autochthonous starter cultures in cheese production – a review. *Food Rev. Int.*, doi: 10.1080/87559129.2022.2097691.

Pavli, F., Argyri, A.A., Skandamis, P., Nychas, G.J., Tassou, C., Chorianopoulos, N. (2019). Antimicrobial activity of oregano essential oil incorporated in sodium alginate edible films: control of *Listeria monocytogenes* and spoilage in ham slices treated with high pressure processing. *Materials*, 12, 3726. doi: 10.3390/ma12223726.

Pereira, C., de Guía Córdoba, M., Aranda, E., Hernández, A., Velázquez, R., Bartolomé, T., Martín, A. (2019). Type of paprika as a critical quality factor in Iberian chorizo sausage manufacture. *CyTA-J. Food*, 17, 907-916. doi: 10.1080/19476337.2019.1668861.

Pérez, M.J., Falqué, E., Domínguez, H. (2016). Antimicrobial action of compounds from marine seaweed. *Mar. Drugs*, 14, 52. doi: 10.3390/md14030052.

Pérez-Ramos, A., Madi-Moussa, D., Coucheney, F., Drider, D. (2021). Current knowledge of the mode of action and immunity mechanisms of LAB-bacteriocins. *Microorganisms*, 9, 2107. doi: 10.3390/Microorganisms9102107.

Pesavento, G., Calonico, C., Bilia, A.R., Barnabei, M., Calesini, F., Addona, R., Mencarelli, L., Carmagnini, L., Di Martino, M.C., Lo Nostro, A. (2015). Antibacterial activity of oregano, rosmarinus and thymus essential oils against *Staphylococcus aureus* and *Listeria monocytogenes* in beef meatballs. *Food Control*, 54, 188-199. doi: 10.1016/j.foodcont.2015.01.045.

Pessione, E., Mazzoli, R., Giuffrida, M.G., Lamberti, C., Garcia-Moruno, E., Barello, C., Conti, A., Giunta, C. (2005). A proteomic approach to studying biogenic amine producing lactic acid bacteria. *Proteomics*, 5, 687-698. doi: 10.1002/ pmic.200401116.

Phasanasophon, K., Kim, S.M. (2018). Antioxidant and cosmeceutical activities of *Agarum cribrosum* phlorotannin extracted by ultrasound treatment. *Nat. Prod. Commun.*, 13, 565-570. doi: 10.1177/1934578X1801300513.

Pingitore, E.V., Todorov, S.D., Sesma, F., Franco, B.D.G.M. (2012). Application of bacteriocinogenic *Enterococcus mundtii* CRL35 and *Enterococcus faecium* ST88Ch in the control of *Listeria monocytogenes* in fresh Minas cheese. *Food Microbiol.*, 32, 38-47. doi: 10.1016/j.fm.2012.04.005.

Pinteus, S., Silva, J., Alves, C., Horta, A., Fino, N., Rodrigues, A.I., Mendes, S., Pedrosa, R. (2017). Cytoprotective effect of seaweeds with high antioxidant activity from the Peniche coast (Portugal). *Food Chem.*, 218, 591-599. doi: 10.1016/j.foodchem.2016.09.067.

Pontonio, E., Montemurro, M., Pinto, D., Marzani, B., Trani, A., Ferrara, G., Mazzeo, A., Gobbetti, M., Rizzello, C.G. (2019). Lactic acid fermentation of pomegranate juice as a tool to improve antioxidant activity. *Front. Microbiol.*, 10, 1-16. doi: 10.3389/fmicb.2019.01550.

Porto, M.C.W., Kuniyoshi, T.M., P.O.S., Vitolo, M., Oliveira, R.P.S. (2017). *Pediococcus* spp.: an important genus of lactic acid bacteria and pediocin producers. *Biotechnol. Adv.*, 35, 361-374. doi: 10.1016/j.biotechadv.2017.03.004.

Pradhan, D., Kadyan, S. (2020). Antifungal lactic acid bacteria (LAB): potential use in food systems. In Mishra, S.K., Goyal M.R. (eds.), *Novel strategies to improve shelf-life and quality of foods*, 1st edition, Apple Academic Press, Palm Bay, USA, Chapter 4, pp. 73-94.

Prado, N., Sampayo, M., González, P., Lombó, F., Díaz, J. (2019). Physicochemical, sensory and microbiological characterization of Asturian Chorizo, a traditional fermented sausage manufactured in Northern Spain. *Meat Sci.*, 156, 118-124. doi: 10.1016/j.meatsci.2019.05.023.

Prakash, B., Kedia, A., Mishra, P.K., Dubey, N.K. (2015). Plant essential oils as food preservatives to control moulds, mycotoxin contamination and oxidative deterioration of agri-food commodities - Potentials and challenges. *Food Control*, 47, 381-391. doi: 10.1016/j.foodcont.2014.07.023.

Puolanne, E., Petaja-Kanninen, E. (2014). Principles of meat fermentation. In Toldrá, F., Hui, Y.H., Astiasarán, I., Sebranek, J.G., Talon, R. (eds.), *Handbook of Fermented Meat and Poultry*, 2nd edition, John Wiley & Sons Inc, Hoboken, USA, pp. 13-17.

Putnik, P., Lorenzo, J.M., Barba, F.J., Roohinejad, S., Jambrak, A.R., Granato, D., Montesano, D., Kovačević, D.B. (2018). Novel food processing and extraction technologies of high-added value compounds from plant materials. *Foods*, 7, 106. doi: 10.3390/foods7070106.

Quirantes-Piné, R., Lozano-Sánchez, J., Herrero, M., Ibáñez, E., Segura-Carretero, A., Fernández-Gutiérrez, A. (2013). HPLC-ESI-QTOFMS as a powerful analytical tool for characterising phenolic compounds in olive-leaf extracts. *Phytochem. Anal.*, 24, 213-223. doi: 10.1002/pca.2401.

R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Radovanović, B.C., Anđelković, A.S.M., Radovanović, A.B., Anđelković, M.Z. (2013). Antioxidant and antimicrobial activity of polyphenol extracts from wild berry fruits grown in southeast Serbia. *Trop. J. Pharm. Res.*, 12, 813-819. doi: 10.4314/tjpr.v12i5.23.

Rafiee, Z., Jafari, S.M., Alami, M., Khomeiri, M. (2011). Microwave-assisted extraction of phenolic compounds from olive leaves; a comparison with maceration. *J. Anim. Plant Sci.*, 21, 738-745.

Raimondi, S., Nappi, M.R., Sirangelo, T.M., Leonardi, A., Amaretti, A., Ulrici, A., Magnani, R., Montanari., C., Tabanelli, G., Gardini., F. (2018). Bacterial community of industrial raw sausage packaged in modified atmosphere throughout the shelf life. *Int. J. Food Microbiol*., 280, 78-86. doi: 10.1016/j.ijfoodmicro.2018.04.041.

Raman, J., Kim, J.S., Choi, K.R., Eun, H., Yang, D., Ko, Y.J., Kim, S.J. (2022). Application of lactic acid bacteria (LAB) in sustainable agriculture: advantages and limitations. *Int. J. Mol. Sci.*, 23, 7784. doi: 10.3390/ijms23147784.

Ramírez, J.A., Castañón-Rodríguez, J.F., Uresti-Marín, R.M. (2021). An exploratory study of possible food waste risks in supermarket fruit and vegetable sections. *Food Sci. Technol.*, 41, 967-973. doi: 10.1590/fst.27320.

Ramírez-Mejía, D., Levers, C., Mas, J.F. (2022). Spatial patterns and determinants of avocado frontier dynamics in Mexico. *Reg. Environ. Chang.*, 22, 28. doi: 10.1007/s10113-022-01883-6.

Rathod, N.B., Ranveer, R.C., Benjakul, S., Kim, S.K., Pagarkar, A.U., Patange, S., Özogul, F. (2021). Recent developments of natural antimicrobials and antioxidants on fish and fishery food products. Compr. Rev. Food Sci. Food Saf., 20, 4182-4210. doi: 10.1111/1541-4337.12787.

Rathod, N.B., Nirmal, N.P., Pagarkar, A., Özogul, F., Rocha, J.M. (2022). Antimicrobial impacts of microbial metabolites on the preservation of fish and fishery products: a review with current knowledge. *Microorganisms*, 10, 773. doi: 10.3390/microorganisms10040773.

Ravyts, F., Barbuti, S., Frustoli, M.A., Parolari, G., Saccani, G., De Vuyst, L., Leroy, F. (2008). Competitiveness and antibacterial potential of bacteriocin-producing starter cultures in different types of fermented sausages. *J. Food Prot.*, 71, 1817-1827. doi: 10.4315/0362-028x-71.9.1817.

Razola-Díaz, M. del C., Guerra-Hernández, E.J., Rodríguez-Pérez, C., Gómez-Caravaca, A.M., García-Villanova, B., Verardo, V. (2021). Optimization of ultrasound-assisted extraction via sonotrode of phenolic compounds from orange by-products. *Foods*, 10, 1120. doi: 10.3390/foods10051120.

Razola-Díaz, M. del C., Gómez-Caravaca, A.M., Guerra-Hernández, E.J., Garcia-Villanova, B., Verardo, V. (2022). new advances in the phenolic composition of Tiger nut (*Cyperus esculentus* L.) by-products. *Foods*, 11, 343. doi: 10.3390/ foods11030343.

Reis, J.A., Paula, A.T., Casarotti, S.N., Penna, A.L.B. (2012). Lactic acid bacteria antimicrobial compounds: Characteristics and applications. Food Eng. Rev., 4, 124-140. doi: 10.1007/s12393-012-9051-2.

Reyes-Jurado, F., Navarro-Cruz, A.R., Ochoa-Velasco, C.E., Palou, E., López-Malo, A., Ávila-Sosa, R. (2020). Essential oils in vapor phase as alternative antimicrobials: a review. *Crit. Rev. Food Sci. Nutr.*, 60, 1641-1650. doi: 10.1080/10408398.2019.1586641.

Riaz, M., Ahmad, M., Rahman, N. (2011). Antimicrobial screening of fruit, leaves, root and stem of *Rubus fruticosus*. *J. Med. Plant Res.*, 5, 5920-5924. doi: 10.5897/JMPR.9000181.

Ribeiro, S.C., O'Connor, P.M., Ross, R.P., Stanton, C., Silva, C.C.G. (2016). An anti-listerial *Lactococcus lactis* strain isolated from Azorean Pico cheese produces lacticin 481. *Int. Dairy J.*, 63, 18-28. doi: 10.1016/j.idairyj.2016.07.017.

Rimaux, T., Vrancken, G., Pothakos, V., Maes, D., De Vuyst, L., Leroy, F. (2011) The kinetics of the arginine deiminase pathway in the meat starter culture *Lactobacillus sakei* CTC 494 are pH-dependent. *Food Microbiol.*, 28, 597-604. doi: 10.1016/j.fm.2010.11.016.

Rincón-Hernández, C.A., De La Pérez, J.L.S., Espinosa-García, F.J. (2011). Caracterización química foliar de los árboles de aguacate criollo (*Persea americana* var. *drymifolia*) en los bancos de germoplasma de Michoacán, México. *Rev. Mex. Biodivers.*, 82, 395-412. doi: 10.22201/ib.20078706e.2011.2.474.

Ritota, M., Manzi, P. (2020). Natural preservatives from plant in cheese making. *Animals*, 10, 749. doi: 10.3390/ani10040749.

Roccato, A., Uyttendaele, M., Barrucci, F., Cibin, V., Favretti, M., Cereser, A., Cin, M.D., Pezzuto, A., Piovesana, A., Longo, A., Ramon, E., De Rui, S., Ricci, A. (2017). Artisanal Italian salami and soppresse: identification of control strategies to manage microbiological hazards. *Food Microbiol.*, 61, 5-13. doi: 10.1016/j.fm.2016.07.010.

Rodríguez-Carpena, J.G., Morcuende, D., Andrade, M.J., Kylli, P., Estévez, M. (2011). Avocado (*Persea americana* Mill.) phenolics, in vitro antioxidant and antimicrobial activities, and inhibition of lipid and protein oxidation in porcine patties. *Agric. Food Chem.*, 59, 5625-5635. doi: 10.1021/jf1048832.

Rodríguez-González, M., Fonseca, S., Centeno, J.A., Carballo J. (2020). Biochemical changes during the manufacture of Galician Chorizo sausage as affected by the addition of autochthonous starter cultures. *Foods*, 9, 1813. doi: 10.3390/foods9121813.

Rojas-García, A., Fuentes, E., Cádiz-Gurrea, M. de la L., Rodriguez, L.; Villegas-Aguilar, M.D.C., Palomo, I., Arráez-Román, D., Segura-Carretero, A. (2022). Biological evaluation of avocado residues as a potential source of bioactive compounds. *Antioxidants*, 11, 1-23. doi: 10.3390/antiox11061049.

Romano, A., Trip, H., Lonvaud-Funel, A., Lolkema, J.S., Lucas, P.M. (2012). Evidence of two functionally distinct ornithine decarboxylation systems in lactic acid bacteria. *Appl. Environ. Microbiol.*, 78, 1953-1961. doi: 10.1128/AEM.07161-11.

Romano, A., Trip, H., Lolkema, J.S., Lucas, P.M. (2013). Three-component lysine/ornithine decarboxylation system in *Lactobacillus saerimneri* 30a. *J. Bacteriol.*, 195, 1249-1254. doi: 10.1128/JB.02070-12.

Rosario, D.K.A., Rodrigues, B.L., Bernardes, P.C., Conte-Junior, C.A. (2020). Principles and applications of non-thermal technologies and alternative chemical compounds in meat and fish. *Crit. Rev. Food Sci. Nutr.*, 61, 1-21. doi: 10.1080/10408398.2020.1754755.

Rossi, F., Rizzotti, L., Felis, G.E., Torriani, S. (2014). Horizontal gene transfer among microorganisms in food: current knowledge and future perspectives. *Food Microbiol.*, 42, 232-243. doi: 10.1016/j.fm.2014.04.004.

Ruiz-Capillas, C., Herrero, A.M. (2019). Impact of biogenic amines on food quality and safety. Foods, 8, 62. doi: 10.3390/foods8020062.

Sabia, C., de Niederhäusern, S., Messi, P., Manicardi, G., Bondi, M. (2003). Bacteriocin-producing *Enterococcus casseliflavus* IM 416K1, a natural antagonist for control of *Listeria monocytogenes* in Italian sausages (cacciatore). *Int. J. Food Microbiol.*, 87, 173-179. doi: 10.1016/s0168-1605(03)00043-6.

Sánchez-Gutiérrez, M., Bascón-Villegas, I., Rodríguez, A., Pérez-Rodríguez, F., Fernández-Prior, Á., Rosal, A., Carrasco, E. (2021). Article valorisation of *Olea europaea* L. olive leaves through the evaluation of their extracts: antioxidant and antimicrobial activity. *Foods*, 10, 966. doi: 10.3390/foods10050966.

Sánchez Mainar, M., Stavropoulou, D., Leroy, F. (2017). Exploring the metabolic heterogeneity of coagulase-negative staphylococci to improve the quality and safety of fermented meats: a review. *Int. J. Food Microbiol.*, 247, 24-37. doi: 10.1016/j.ijfoodmicro.2016.05.021.

Sánchez-Maldonado, A.F., Schieber, A., Gänzle, M.G. (2011). Structure-function relationships of the antibacterial activity of phenolic acids and their metabolism by lactic acid bacteria. *J. Appl. Microbiol.*, 111, 1176-1184. doi: 10.1111/j.1365-2672.2011.05141.x.

Santana-Méridas, O., González-Coloma, A., Sánchez-Vioque, R. (2012). Agricultural residues as a source of bioactive natural products. *Phytochem. Rev.*, 11, 447-466. doi: 10.1007/s11101-012-9266-0.

Sanyal, S., Chatterjee, A. (2021). Antimicrobial action and biopreservation by autochthonous lactic acid bacteria of fish. *Adv. zool. bot.*, 9, 37-44. doi: 10.13189/azb.2021.090201.

Saraoui, T., Cornet, J., Guillouet, E., Pilet, M.F., Chevalier, F., Joffraud, J.J., Leroi, F. (2017). Improving simultaneously the quality and safety of cooked and peeled shrimp using a cocktail of bioprotective lactic acid bacteria. *Int. J. Food Microbiol.*, 241, 69-77. doi: 10.1016/j.ijfoodmicro.2016.09.024.

Savran, D., Pérez-Rodríguez, F., Halkman, A.K. (2018). Modelling survival of *Salmonella enteritidis* during storage of yoghurt at different temperatures. *Int. J. Food Microbiol.*, 271, 67-76. doi: 10.1016/j.ijfoodmicro.2018.02.017.

Scatassa, M.L., Gaglio, R., Cardamone, C., Macaluso, G., Arcuri, L., Todaro, M., Mancuso, I. (2017). Anti-listeria activity of lactic acid bacteria in two traditional Sicilian cheeses. *Ital. J. Food Saf.*, 6, 6191. doi: 10.4081/ijfs.2017.6191.

Selma, M.V., Espín, J.C., Tomás-Barberán, F.A. (2009). Interaction between phenolics and gut microbiota: role in human health. *J. Agric. Food Chem.*, 57, 6485-6501. doi: 10.1021/jf902107d.

Semerdjieva, I.B., Zheljazkov, V.D., Dincheva, I., Astatkie, T., Kačániová, M. (2020). Chemotypes of *Juniperus oxycedrus* in Bulgaria and the antimicrobial activity of galbuli essential oils. *Ind. Crops Prod.*, 158, 113005. doi: 10.1016/j.indcrop.2020.113005.

Serra-Castelló, C., Costa, J.C.C.P., Jofré, A., Bolívar, A., Pérez-Rodríguez, F., Bover-Cid, S. (2022). A mathematical model to predict the antilisteria bioprotective effect of *Latilactobacillus sakei* CTC494 in vacuum packaged cooked ham. *Int. J. Food Microbiol.*, 363, 109491. doi: 10.1016/j.ijfoodmicro.2021.109491.

Sharifi-Rad, J., Sureda, A., Tenore, G.C., Daglia, M., Sharifi-Rad, M., Valussi, M., Tundis, R., Sharifi-Rad, M., Loizzo, M.R., Ademiluyi, A.O., Sharifi-Rad, R., Ayatollahi, S.A, Iriti, M. (2017). Biological activities of essential oils: from plant chemoecology to traditional healing systems. *Molecules*, 22, 70. doi: 10.3390/ molecules22010070.

Sahin, S., Samlı, R. (2013). Optimization of olive leaf extract obtained by ultrasound-assisted extraction with response surface methodology. *Ultrason. Sonochem.*, 20, 595-602. doi: 10.1016/j.ultsonch.2012.07.029.

Sheng, L., Wang, L. (2021). The microbial safety of fish and fish products: recent advances in understanding its significance, contamination sources, and control strategies. *Compr. Rev. Food Sci. Food Saf.*, 20, 738-786. doi: 10.1111/1541-4337.12671.

Sibanda, T., Buys, E.M. (2017). Resuscitation and growth kinetics of sub-lethally injured Listeria monocytogenes strains following fluorescence activated cell sorting (FACS). *Food Res. Int.*, 100, 150-158. doi: 10.1016/j.foodres.2017.08.020.

Siddi, G., Piras, F., Spanu, V., Meloni, M.P., Sanna, R., Carta, N., Errico, M., Cuccu, M., De Santis, E.P.L., Scarano, C. (2022). Selection of commercial protective cultures to be added in Sardinian fermented sausage to control *Listeria monocytogenes*. *Ital. J. Food Saf.*, 11, 10368. doi: 10.4081/ijfs.2022.10368.

Silva Ferrari, I., de Souza, J.V., Ramos, C.L., da Costa, M.M., Schwan, R.F., Dias, F.S. (2016). Selection of autochthonous lactic acid bacteria from goat dairies and their addition to evaluate the inhibition of *Salmonella typhi* in artisanal cheese. *Food Microbiol.*, 60, 29-38. doi: 10.1016/j.fm.2016.06.014.

Simons, A., Alhanout, K., Duval, R.E. (2020). Bacteriocins, antimicrobial peptides from bacterial origin: overview of their biology and their impact against multidrug-resistant bacteria. *Microorganisms*, 8, 639, doi:10.3390/microorganisms8050639.

Sindelar, J.J., Milkowski, A.L. (2012). Human safety controversies surrounding nitrate and nitrite in the diet. *Nitric Oxide*, 26, 259-266. doi: 10.1016/j.niox.2012.03.011.

Singh, V.P. (2018). Recent approaches in food bio-preservation - A review. *Open Vet. J.*, 8, 104-111. doi: 10.4314/ovj.v8i1.16.

Singh, A.K., Kim, J.Y., Lee, Y.S. (2022) Phenolic compounds in active packaging and edible films/coatings: natural bioactive molecules and novel packaging ingredients. *Molecules*, 27, 7513. doi: 10.3390/molecules27217513.

Siroli, L., Patrignani, F., Serrazanetti, D.I., Tabanelli, G., Montanari, C., Gardini, F., Lanciotti, R. (2015). Lactic acid bacteria and natural antimicrobials to improve the safety and shelf-life of minimally processed sliced apples and lamb's lettuce. *Food Microbiol.*, 47, 74-84. doi: 10.1016/j.fm.2014.11.008.

Skroza, D. Šimat, V., Smole Možina, S., Katalinić, V., Boban, N., Generalić Mekinić, I. (2019). Interactions of resveratrol with other phenolics and activity against food-borne pathogens. *Food Sci. Nutr.*, 7, 2312-2318. doi: 10.1002/fsn3.1073.

Smid, E.J., Kleerebezem, M. (2014). Production of aroma compounds in lactic fermentations. *Annu. Rev. Food Sci. Technol.*, 5, 313-326. doi: 10.1146/annurev-food-030713-092339.

Solís-Salas, L.M., Sierra-Rivera, C.A., Cobos-Puc, L.E., Ascacio-Valdés, J.A., Silva-Belmares, S.Y. (2021). Antibacterial potential by rupture membrane and antioxidant capacity of purified phenolic fractions of *Persea americana* leaf extract. *Antibiotics*, 10, 508. doi: 10.3390/antibiotics10050508.

Soltani, S., Hammami, R., Cotter, P.D., Rebuffat, S., Ben Said, S., Gaudreau, H., Bédard, F., Biron, E., Drider, D., Fliss, I. (2021). Bacteriocins as a new generation of antimicrobials: toxicity aspects and regulations. *FEMS Microbiol. Rev.*, 45, 1-24. doi: 10.1093/femsre/fuaa039.

Sommaruga, R., Eldridge, H.M. (2021). Avocado production: water footprint and socio-economic implications. *EuroChoices*, 20, 48-53. doi: 10.1111/1746-692X.12289.

Soto, M.L., Moure, A., Domínguez, H., Parajó, J.C. (2011). Recovery, concentration and purification of phenolic compounds by adsorption: a review. *J. Food Eng.*, 105, 1-27. doi: 10.1016/j.jfoodeng.2011.02.010.

Spitaels, F., Wieme, A.D., Janssens, M., Aerts, M., Daniel, H.M., Van Landschoot, A., De Vuyst L., Vandamme P. (2014). The microbial diversity of traditional spontaneously fermented lambic beer. *PLoS ONE*, 9, e95384. doi: 10.1371/journal.pone.0095384.

Stanojevic-Nikolic, S., Dimic, G., Mojovic, L., Pejin, J., Djukic-Vukovic, A., Kocic-Tanackov, S. (2015). Antimicrobial activity of lactic acid against pathogen and spoilage microorganisms. J. *Food Processing Preserv.*, 40, 990-998. doi: 10.1111/jfpp.12679.

Starzyńska-Janiszewska, A., Fernández-Fernández, C., Martín-García, B., Verardo, V., Gómez-Caravaca, A.M. (2022). Solid state fermentation of olive leaves as a promising technology to obtain hydroxytyrosol and elenolic acid derivatives enriched extracts. *Antioxidants*, 11, 1693. doi: 10.3390/antiox11091693.

Sukor, N., Jusoh, R., Rahim, S.A., Kamarudin, N. (2018). Ultrasound assisted methods for enhanced extraction of phenolic acids from *Quercus Infectoria* galls. *Mater. Today Proc.*, 5, 21990-21999. doi. 10.1016/j.matpr.2018.07.060.

Sutili, F.J., Gatlin, D.M., Heinzmann, B.M., Baldisserotto, B. (2018). Plant essential oils as fish diet additives: Benefits on fish health and stability in feed. *Rev. Aquac.*, 10, 716-726. doi: 10.1111/raq.12197.

Tabanelli, G., Montanari, C., Grazia, L., Lanciotti, R., Gardini, F. (2013). Effects of a_w at packaging time and atmosphere composition on aroma profile, biogenic amine content and microbiological features of dry fermented sausages. *Meat Sci.*, 94, 177-186. doi: 10.1016/j.meatsci.2013.01.018.

Tabanelli, G., Montanari, C., Patrignani F., Siroli, L., Lanciotti, R., Gardini, F. (2014). Modeling with the logistic regression the growth/no growth interface of Saccharomyces cerevisiae in relation to two antimicrobial terpenes (citral and linalool), pH and a_w. *J. Food Sci.*, 79, M391-M398. doi: 10.1111/1750-3841.12369.

Tabanelli, G., Barbieri, F., Campedelli, I., Venturini, M.C., Gardini, F., Montanari, C. (2020). Effects of bioprotective cultures on the microbial community during storage of Italian fresh filled pasta. Food Control, 115, 107304. doi: 10.1016/j.foodcont.2020.107304.

Tabanelli, G., Barbieri, F., Soglia, F., Magnani, R., Gardini, G., Petracci, M., Gardini, F., Montanari, C. (2022). Safety and technological issues of dry fermented sausages produced without nitrate and nitrite. *Food Res. Int.*, 160, 111685. doi: 10.1016/j.foodres.2022.111685.

Tahiri, I., Desbiens, M., Kheadr, E., Lacroix, C., Fliss, I. (2009). Comparison of different application strategies of divergicin M35 for inactivation of *Listeria monocytogenes* in cold-smoked wild salmon. *Food Microbiol.*, 26, 783-793. doi: 10.1016/j.fm.2009.05.003.

Tagg, J.R., McGiven, A.R. (1971). Assay system for bacteriocins. *Appl. Microbiol.*, 21, 943. doi: 10.1128/am.21.5.943-943.1971.

Talhaoui, N., Gómez-Caravaca, A.M., Roldan, C., León, L., De la Rosa, R., Fernández-Gutiérrez, A., Segura-Carretero, A. (2015). Chemometric analysis for the evaluation of phenolic patterns in olive leaves from six cultivars at different growth stages. *J. Agric. Food Chem.*, 63, 1722-1729. doi: 10.1021/jf5058205.

Talon, R., Leroy, S. (2011). Diversity and safety hazards of bacteria involved in meat fermentations. *Meat Sci.*, 89, 303-309. doi: 10.1016/j.meatsci.2011.04.029.

Talon, R., Leroy, S., Lebert, I., Giammarinaro, P., Chacornac, J.P., Latorre-Moratalla, M., Vidal-Carou, C., Zanardi, E., Conter, M., Lebecque, A. (2008). Safety improvement and preservation of typical sensory qualities of traditional dry fermented sausages using autochthonous starter cultures. *Int. J. Food Microbiol.*, 126, 227-234. doi: 10.1016/j.ijfoodmicro.2008.05.031.

Tamasiga, P., Miri, T., Onyeaka, H., Hart, A. (2022). Food waste and circular economy: challenges and opportunities. *Sustainability*, 14, 9896. doi: 10.3390/su14169896.

Tattershall, E., Nenadic, G., Stevens, R.D. (2021). Modelling trend life cycles in scientific research using the Logistic and Gompertz equations. *Scientometrics*, 126, 9113-9132. doi: 10.1007/s11192-021-04137-0.

Taviano, M.F., Marino, A., Trovato, A., Bellinghieri, V., Melchini, A., Dugo, P., Cacciola, F., Donato, P., Mondello, L., Güvenç, A., De Pasquale, R., Miceli, N. (2013). *Juniperus oxycedrus* L. subsp. *oxycedrus* and *Juniperus oxycedrus* L. subsp. *macrocarpa* (Sibth. & Sm.) Ball. "berries" from Turkey: comparative evaluation of phenolic profile, antioxidant, cytotoxic and antimicrobial activities. *Food Chem. Toxicol.*, 58, 22-29. doi: 10.1016/j.fct.2013.03.049.

Tchatchouang, C.D.K., Fri, J., De Santi, M., Brandi, G., Schiavano, G.F., Amagliani, G., Ateba, C.N. (2020). Listeriosis outbreak in South Africa: a comparative analysis with previously reported cases worldwide. *Microorganisms*, 8, 135 doi: 10.3390/microorganisms8010135.

Terpou, A., Bosnea, L., Kanellaki, M., Plessas, S., Bekatorou, A., Bezirtzoglou, E., Koutinas, A.A. (2018). Growth capacity of a novel potential probiotic *Lactobacillus paracasei* K5 strain incorporated in industrial white brined cheese as an adjunct culture. *J. Food Sci.*, 83, 723-731. doi: 10.1111/1750-3841.14079.

Todorov, S.D., Stojanovski, S., Iliev, I., Moncheva, P., Nero, L.A., Ivanova, I.V. (2017). Technology and safety assessment for lactic acid bacteria isolated from traditional Bulgarian fermented meat product 'Lukanka'. *Braz. J. Microbiol.*, 48, 576-586. doi: 10.1016/j.bjm.2017.02.005.

Todorov, S.D., Dioso, C.M., Liong, M.T., Vasileva, T., Moncheva, P., Ivanova, I.V., Iliev, I. (2022). Lukanka, a semi-dried fermented traditional Bulgarian sausage: role of the bacterial cultures in its technological, safety and beneficial characteristics. *Appl. Food Biotechnol.*, 9, 255-265. doi: 10.22037/afb.v9i4.38488.

Toldrá, F., Hui, Y.H. (2014). Dry-fermented sausages and ripened meats: an overview. In Toldrá, F., Hui, Y.H., Astiasarán, I., Sebranek, J.G., Talon, R. (eds.), *Handbook of Fermented Meat and Poultry*, 2nd edition, John Wiley & Sons Inc, Hoboken, USA, pp. 1-6.

Tomović, V., Šojić, B., Savanović, J., Kocić-Tanackov, S., Pavlić, B., Jokanović, M., Đorđević, V., Parunović, N., Martinović, A., Vujadinović, D. (2020). New formulation towards healthier meat products: *Juniperus communis* L. essential oil as alternative for sodium nitrite in dry fermented sausages. *Foods*, 9, 1066. doi: 10.3390/foods9081066.

Tosun, Ş.Y., Üçok Alakavuk, D., Ulusoy, Ş., & Erkan, N. (2018). Effects of essential oils on the survival of *Salmonella* Enteritidis and *Listeria monocytogenes* on fresh Atlantic salmons (*Salmo salar*) during storage at 2 ± 1°C. *J. Food Saf.*, 38, e12408. doi: 10.1111/jfs.12408.

Trujillo-Mayol, I., Badillo-Muñoz, G., Céspedes-Acuña, C., Alarcón-Enos, J. (2020). The relationship between fruit size and phenolic and enzymatic composition of avocado by-products (*Persea americana* Mill.): the importance for biorefinery applications. *Horticulturae*, 6, 91. doi: 10.3390/horticulturae6040091.

Van Haute S., Raes K., Van der Meeren, P., & Sampers, I. (2016). The effect of cinnamon, oregano and thyme essential oils in marinade on the microbial shelf life of fish and meat products. *Food Control*, 68, 30-39. doi: 10.1016/j.foodcont.2016.03.025.

Van Reckem, E., Geeraerts, W., Charmpi, C., Van der Veken, D., De Vuyst L., Leroy F. (2019). Exploring the link between the geographical origin of European fermented foods and the diversity of their bacterial communities: the case of fermented meats. *Front. Microbiol.*, 10, 2302. doi: 10.3389/fmicb.2019.02302.

Van Reckem, E., Claeys, E., Charmpi, C., Fajardo, A.S., Van der Veken, D., Maes, D., Weckx, S., De Vuyst, L., Leroy, F. (2021). High-throughput amplicon sequencing to assess the impact of processing factors on the development of microbial communities during spontaneous meat fermentation. *Int. J. Food Microbiol.*, 354, 109322 doi: 10.1016/j.ijfoodmicro.2021.109322.

Vasilijević, B., Mitić-Culafi, D., Djekic, I., Marković, T., Knežević-Vukčević, J., Tomasevic, I., Velebit, B., Nikolić, B. (2019). Antibacterial effect of *Juniperus communis* and *Satureja montana* essential oils against *Listeria monocytogenes* in vitro and in wine marinated beef. *Food Control*., 100, 247-256. doi: 10.1016/j.foodcont.2019.01.025.

Vaz-Velho, M., Jácome, S., Noronha, L., Todorov, S., Fonseca, S., Pinheiro, R., Morais, A., Silva, J., Teixeira, P. (2013). Comparison of antilisterial effects of two strains of lactic acid bacteria during processing and storage of a Portuguese salami-like product '*alheira*'. *Chem. Eng. Trans.*, 32, 1807-1812. doi: 10.3303/CET1332302.

Veličković, I., Živković, J., Stojković, D., Sokovic, M.D., Marin, P.D., Grujić, S. (2021). Evaluation of antioxidant, antimicrobial and potential food preserving properties of *Rubus discolor* (*Rosaceae*) fruit extracts. *Nat. Prod. Commun.*, 16, 1-9. doi: 10.1177/1934578X211009692.

Vergis, J., Gokulakrishnan, P., Agarwal, R.K., Kumar, A. (2015). Essential oils as natural food antimicrobial agents: a review. *Crit. Rev. Food Sci. Nutr.*, 55, 1320-1323. doi: 10.1080/10408398.2012.692127.

Verma, R., Gangrade, T., Punasiya, R., Ghulaxe, C. (2014). *Rubus fruticosus* (blackberry) use as an herbal medicine. *Pharmacogn. Rev.*, 8, 101. doi: 10.4103/0973-7847.134239.

Verni, M., De Mastro, G., De Cillis, F., Gobbetti, M., Rizzello, C.G. (2019). Lactic acid bacteria fermentation to exploit the nutritional potential of Mediterranean faba bean local biotypes. *Food Res. Int.*, 125, 108571. doi: 10.1016/j.foodres.2019.108571.

Verni, M., Pontonio, E., Krona, A., Jacob, S., Pinto, D., Rinaldi, F., Verardo, V., Díaz-de-Cerio, E., Coda, R., Rizzello, C.G. (2020). Bioprocessing of brewers' spent grain enhances its antioxidant activity: characterization of phenolic compounds and bioactive peptides. *Front. Microbiol.*, 11, 1-15. doi: 10.3389/fmicb.2020.01831.

Vijayan, A., Sivaraman, G.K., Visnuvinayagam, S., Mothadaka, M.P. (2021). Role of natural additives on quality and shelf life extension of fish and fishery products. In Lage, M.A.A.P., Otero, P. (eds.), *Food Additives,* Intech Open, London, UK.

Wang, C., Chang, T., Yang, H., Cui, M. (2015). Antibacterial mechanism of lactic acid on physiological and morphological properties of *Salmonella enteritidis*, *Escherichia coli* and *Listeria monocytogenes*. *Food Control*, 47, 231-236. doi: 10.1016/j.foodcont.2014.06.034.

Wang, W., Xia, W., Gao, P., Xu, Y., & Jiang, Q. (2017). Proteolysis during fermentation of Suanyu as a traditional fermented fish product of China. *Int. J. Food Prop.*, 20, S166-S176. doi: 10.1080/10942912.2017.1293089.

Wang, H., Wang, H., Li, D., Luo, Y. (2018). Effect of chitosan and garlic essential oil on microbiological and biochemical changes that affect quality in grass carp (*Ctenopharyngodon idellus*) fillets during storage at 4°C. *Aquat. Food Prod. Technol.*, 27, 80-90. doi: 10.1080/10498850.2017.1403525.

Wang, Y., Wu, J., Lv, M., Shao, Z., Hungwe, M., Wang, J., Bai, X., Xie, J., Wang, Y., Geng, W. (2021). Metabolism characteristics of lactic acid bacteria and the expanding applications in food industry. *Front. Bioeng. Biotechnol.*, 9, 1-19. doi: 10.3389/fbioe.2021.612285.

Wajs-Bonikowska, A., Stobiecka, A., Bonikowski, R., Krajewska, A., Sikora, M., Kula, J. (2017). A comparative study on composition and antioxidant activities of supercritical carbon dioxide, hexane and ethanol extracts from blackberry (*Rubus fruticosus*) growing in Poland. *J. Sci. Food Agric.*, 97, 3576-3583. doi: 10.1002/jsfa.8216.

WHO (World Health Organization). (2014). Antimicrobial resistance: global report on surveillance. World Health Organization, Geneva. https://apps.who.int/iris/handle/10665/112642.

Widenmann, A., Schiffer, C., Ehrmann, M., Vogel, R. (2022). Impact of different sugars and glycosyltransferases on the assertiveness of *Latilactobacillus sakei* in raw sausage fermentations. *Int. J. Food Microbiol.*, 366, 109575. doi: 10.1016/j.ijfoodmicro.2022.109575.

Wiernasz, N., Cornet, J., Cardinal, M., Pilet, M.F., Passerini, D., Leroi, F. (2017). Lactic acid bacteria selection for biopreservation as a part of hurdle technology approach applied on seafood. Front. Mar. Sci., 4, 119. doi: 10.3389/fmars.2017.00119.

Wiernasz, N., Leroi, F., Chevalier, F., Cornet, J., Cardinal, M., Rohloff, J., Passerini, D., Skirnisdóttir, S., Pilet, M.F. (2020). Salmon gravlax biopreservation with lactic acid bacteria: a polyphasic approach to assessing the impact on organoleptic properties, microbial ecosystem and volatilome composition. *Front. Microbiol.*, 10, 3103. doi: 10.3389/fmicb.2019.03103.

Wijesinghe, W.A.J.P., Jeon, Y.J. (2012). Exploiting biological activities of brown seaweed *Ecklonia cava* for potential industrial applications: a review. *Int. J. Food Sci. Nutr.*, 63, 225-235. doi: 10.3109/09637486.2011.619965.

Wójcik, W., Łukasiewicz, M., Puppel, K. (2021). Biogenic amines: formation, action and toxicity–a review. *J. Sci. Food Agric.*, 101, 2634-2640. doi: 10.1002/jsfa.10928.

Woraprayote, W., Malila, Y., Sorapukdee, S., Swetwiwathana, A., Benjakul, S., Visessanguan, W. (2016). Bacteriocins from lactic acid bacteria and their applications in meat and meat products. *Meat Sci.*, 120, 118-132. doi: 10.1016/j.meatsci.2016.04.004.

Wunderlichová, L., Buňková, L., Koutný, M., Jančová, P., Buňka, F. (2014). Formation, degradation, and detoxification of putrescine by foodborne bacteria: a review. *Compr. Rev. Food Sci. Food Saf.*, 13, 1012-1033. doi: 10.1111/1541-4337.12099.

Xiong, Y., Zhang, P., Warner, R.D., Shen, S., Johnson, S., Fang, Z. (2020). HPLC-DAD-ESI-QTOF-MS/MS qualitative analysis data and HPLC-DAD quantification data of phenolic compounds of grains from five Australian sorghum genotypes. *Data Br.*, 33, 106584. doi: 10.1016/J.DIB.2020.106584.

Xu, P., Xu, X.B., Khan, A., Fotina, T., Wang, S.H. (2021). Antibiofilm activity against *Staphylococcus aureus* and content analysis of *Taraxacum Officinale* phenolic extract. *Pol. J. Vet. Sci.*, 24, 243-251. doi: 10.24425/pjvs.2021.137659.

Xu, Y., Zang, J., Regenstein, J.M., Xia, W. (2021). Technological roles of microorganisms in fish fermentation: a review. *Crit. Rev. Food Sci. Nutr.*, 61, 1000-1012. doi: 10.1080/10408398.2020.1750342.

Yamassaki, F.T., Campestrini, L.H., Faria, S., Bello, J., Maurer, B. (2017). Avocado leaves: Influence of drying process, thermal incubation, and storage conditions on preservation of polyphenolic compounds and antioxidant activity. *Int. J. Food Prop.*, 20, 2280-2293. doi: 10.1080/10942912.2017.1369105.

Yamassaki, F.T., Campestrini, L.H., Zawadzki-Baggio, S.F., Maurer, J.B.B. (2018). Chemical characterization and complement modulating activities of an arabinogalactan-protein-rich fraction from an aqueous extract of avocado leaves. *Int. J. Biol. Macromol.*, 120, 513-521. doi: 10.1016/J.IJBIOMAC.2018.08.072.

Yang, H., Hewes, D., Salaheen, S., Federman, C., Biswas, D. (2014). Effects of blackberry juice on growth inhibition of foodborne pathogens and growth promotion of *Lactobacillus*. *Food Control*, 37, 15-20. doi: 10.1016/j.foodcont.2013.08.042.

Yang, E., Fan, L., Yan, J. Jiang, Y., Doucette, C., Fillmore, S., Walker B. (2018). Influence of culture media, pH and temperature on growth and bacteriocin production of bacteriocinogenic lactic acid bacteria. *Amb. Express*, 8, 10. doi: 10.1186/s13568-018-0536-0.

Yap, P.C., MatRahim, N.A., AbuBakar, S., Lee, H.Y. (2021). Antilisterial potential of lactic acid bacteria in eliminating *Listeria monocytogenes* in host and ready-to-eat food application. *Microbiol. Res.*, 12, 234-257. doi: 10.3390/Microbiolres12010017.

Yeriikaya, O., Akan, E., Bayram, O.Y., Karaman, A.D., Kinik, O. (2021). The impact of medicinal and aromatic plant addition on antioxidant, total phenolic, antimicrobial activities, and microbiological quality of Mozzarella cheese. Int. Food Res. J., 28, 508-516. doi:10.47836/ifrj.28.3.10.

Yousefi, M., Khorshidian, N., Hosseini, H. (2020). Potential application of essential oils for mitigation of *Listeria monocytogenes* in meat and poultry products. *Front. Nutr.*, 7, 577287. doi: 10.3389/fnut.2020.577287.

Yu, D., Xu, Y., Jiang, Q., Xia, W. (2017). Effects of chitosan coating combined with essential oils on quality and antioxidant enzyme activities of grass carp (*Ctenopharyngodon idellus*) fillets stored at 4°C. *Int. J. Food Sci. Technol.*, 52, 404-412. doi: 10.1111/ijfs.13295.

Zacharof, M.P., Lovitt, R.W. (2012). Bacteriocins produced by lactic acid bacteria a review article. *APCBEE Procedia*, 2, 50-56. doi: 10.1016/j.apcbee.2012.06.010.

Zafar, T., Sidhu, J.S. (2018). Avocado production, processing, and nutrition. In Siddiq, M., Uebersax, M.A. (eds.), *Handbook of Vegetables and Vegetable Processing*, 2nd edition, John Wiley & Sons Inc, Hoboken, USA, pp. 509-534.

Zaman, M.Z., Bakar, F.A., Selamat, J., Bakar, J., Ang, S.S., Chong, C.Y. (2014). Degradation of histamine by the halotolerant *Staphylococcus carnosus* FS19 isolate obtained from fish sauce. *Food Control*, 40, 58-63. doi: 10.1016/j.foodcont.2013.11.031.

Zamuz, S., Munekata, P.E.S., Dzuvor, C.K.O., Zhang, W., Sant'Ana, A.S., Lorenzo, J.M. (2021). The role of phenolic compounds against *Listeria monocytogenes* in food. A review. *Trends Food Sci. Technol*., 110, 385-392. doi: 10.1016/j.tifs.2021.01.068.

Zang, J., Xu, Y., Xia, W., Regenstein, J. M. (2020). Quality, functionality, and microbiology of fermented fish: a review. *Crit. Rev. Food Sci. Nutr.*, 60, 1228-1242. doi: 10.1080/10408398.2019.1565491.

Zapaśnik, A., Sokołowska, B., Bryła, M. (2022). Role of lactic acid bacteria in food preservation and safety. *Foods*, 11, 1283. doi: 10.3390/foods11091283.

Zhang, H.F., Yang, X.H., Wang, Y. (2011). Microwave assisted extraction of secondary metabolites from plants: current status and future directions. *Trends Food Sci. Technol.*, 22, 672-688. doi: 10.1016/j.tifs.2011.07.003.

Zhang, Y., Li, D., Lv, J., Li, Q., Kong, C., Luo, Y. (2017a). Effect of cinnamon essential oil on bacterial diversity and shelf-life in vacuum-packaged common carp (*Cyprinus carpio*) during refrigerated storage. *Int. J. Food Microbiol.*, 249, 1-8. doi: 10.1016/j.ijfoodmicro.2016.10.008.

Zhang, Y., Chen, J., Wang, L., Cao, J., Xu, L. (2017b). Chemical composition and biological activities of the essential oil from *Rubus pungens* var. *oldhamii*. *Nat. Prod. Res.*, 31, 1454-1458. doi: 10.1080/14786419.2016.1253076.

Zhang, C., Chen, X., Li, Y., Ding, W., Fu, G. (2018a). Water-energy-food nexus: concepts, questions and methodologies. *J. Clean. Prod.*, 195, 625-639. doi: 10.1016/j.jclepro.2018.05.194.

Zhang, Q.W., Lin, L.G., Ye, W.C. (2018b). Techniques for extraction and isolation of natural products: a comprehensive review. *Chin. Med.*, 13, 20. doi: 10.1186/s13020-018-0177-x.

Zhao, Y., Wen, C., Feng, Y., Zhang, J., He, Y., Duan, Y., Zhang, H., Ma, H. (2021). Effects of ultrasound-assisted extraction on the structural, functional and antioxidant properties of *Dolichos lablab* L. *Protein. Process Biochem.*, 101, 274-284. doi: 10.1016/j.procbio.2020.11.027.

Živić, N., Milošević, S., Dekić, V., Dekić, B., Ristić, N., Ristić, M., Sretić, L. (2019). Phytochemical and antioxidant screening of some extracts of *Juniperus communis* L. and *Juniperus oxycedrus* L. *Czech J. Food Sci.*, 37, 351-358. doi: 10.17221/28/2019-CJFS.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M., van't Riet, K. (1990). Modeling of the bacterial growth curve. *Appl. Environ. Microbiol.*, 56, 1875-1881. doi: 10.1128/aem.56.6.1875-1881.1990.