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Director: Prof. Valentino Bontempo

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Improvement of food safety and microbial interactions

Erica Tirloni

Tutor:

Prof. Patrizia Cattaneo

Coordinator:

Prof. Giovanni Savoini

Cover images: inhibition halos of *Serratia marcescens* by *Lactobacillus animalis* SB310 (left) and *Pseudomonas aeruginosa* by the mixture *Lactobacillus animalis* SB310-*Lactobacillus paracasei* subsp. *paracasei* SB137 (1:1) (right).

Index

1.	Foreword	11
1.0	Microbial ecology of food products	13
1.1	Food spoilage	13
1.2	Microbial interactions	18
1.3	Specific Spoilage Organisms (SSOs) and fish spoilage	22
	<i>1.3.1 Spoilage of fish and fish products</i>	22
	<i>1.3.1.1 Initial microflora</i>	22
	<i>1.3.1.2 Spoilage of fish products</i>	24
	<i>1.3.2 Specific Spoilage Organisms (SSOs)</i>	26
1.4	Ephemeral spoilage organisms (ESOs) and meat spoilage	28
	<i>1.4.1 Spoilage of meat and meat products</i>	28
	<i>1.4.2 Ephemeral spoilage organisms (ESOs)</i>	31
1.5	Preservation of fish	34
	<i>1.5.1 Low temperature storage</i>	34
	<i>1.5.2 Controlling water activity</i>	35
	<i>1.5.3 Controlling autolytic enzymatic spoilage</i>	35
	<i>1.5.4 Control of oxidative spoilage through phenolic antioxidants and Ethylenediaminetetraacetic Acid (EDTA)</i>	35
	<i>1.5.5 Use of Antimicrobials</i>	36
	<i>1.5.6 Biopreservation by Lactic Acid Bacteria</i>	37
1.6	Meat preservation	40
	<i>1.6.1 Cold temperatures</i>	40
	<i>1.6.2 Chemical preservatives</i>	41
	<i>1.6.3 Ionising radiations</i>	41
	<i>1.6.4 High hydrostatic pressure</i>	42
	<i>1.6.5 Packaging</i>	42
	<i>1.6.6 Thermal alternative technologies</i>	44
	<i>1.6.7 Biopreservation</i>	44
1.7	References	47
2.	Objectives	63
2.1	References	66

3.	Effect of the Lactic Acid Bacteria on the control of listerial activity and shelf-life of smoked salmon scraps	69
3.1	Abstract	71
3.2	Introduction	71
3.3	Materials and methods	73
	3.3.1 <i>Scraps samples</i>	73
	3.3.2 <i>Experimental design</i>	73
	3.3.3 <i>Microbiological analyses</i>	73
	3.3.4 <i>Identification of LAB and L. monocytogenes strains</i>	74
	3.3.5 <i>Anti-listerial activity</i>	75
	3.3.6 <i>Physical and chemical analyses</i>	75
	3.3.7 <i>Solid – Phase Microextraction (SPME) Headspace analysis</i>	76
	3.3.8 <i>Gas Chromatography-Mass spectrometry (GC/MS) analysis</i>	76
	3.3.9 <i>Statistical analysis</i>	76
3.4	Results	77
	3.4.1 <i>Identification of LAB strains from cold smoked salmon scraps</i>	77
	3.4.2 <i>Anti-listerial activity</i>	78
	3.4.3 <i>Microbiological analyses and identification of L. monocytogenes isolates</i>	79
	3.4.4 <i>Physicochemical characteristics</i>	82
	3.4.5 <i>Volatile compounds</i>	82
	3.4.6 <i>Shelf-life</i>	84
3.5	Conclusions	84
3.6	Acknowledgements	85
3.7	References	85
4.	<i>In vitro</i> evaluation of <i>Lactobacillus animalis</i> SB310, <i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> SB137 and their mixtures as potential bioprotective agents for raw meat	91
4.1	Abstract	93
4.2	Introduction	94
4.3	Materials and methods	95
	4.3.1 <i>Preparation of Lactobacillus strains</i>	95
	4.3.2 <i>Antimicrobial activity against spoilage and potential pathogenic microorganisms - First Trial</i>	95
	4.3.3 <i>Antimicrobial activity of cell-free supernatants against spoilage and potential pathogenic microorganisms</i>	96
	4.3.4 <i>Second trial</i>	97
	4.3.5 <i>Statistical analyses</i>	97

4.4	Results	98
4.4.1	<i>Trial 1 - Antimicrobial activity of viable Lactobacilli against spoilage and potential pathogenic microorganisms</i>	98
4.4.2	<i>Trial 2 - Antimicrobial activity of different mixtures of viable Lactobacilli</i>	101
4.4.3	<i>Antimicrobial activity of cell-free supernatants of single strains and the mixtures (1:1a, 1:1b, 1:5, 1:10, 2:1, 5:1, 10:1)</i>	103
4.5	Conclusions	105
4.6	Acknowledgements	105
4.7	References	105
5.	Evaluation of the <i>in vitro</i> antimicrobial activity of mixtures of <i>Lactobacillus sakei</i> and <i>L. curvatus</i> isolated from Argentine meat and their application on vacuum-packed beef	109
5.1	Abstract	111
5.2	Introduction	112
5.3	Materials and methods	113
5.3.1	<i>Preparation of Lactobacillus strains</i>	113
5.3.2	<i>Antimicrobial activity against spoilage and potential pathogenic microorganisms</i>	114
5.3.3	<i>Antimicrobial activity of cell-free supernatants against spoilage and potential pathogenic microorganisms</i>	114
5.3.4	<i>Preparation and inoculation of vacuum-packaged meat slices</i>	115
5.3.5	<i>Microbiological analyses</i>	116
5.3.6	<i>Physical and chemical analyses</i>	116
5.3.7	<i>Statistical analysis</i>	116
5.4	Results and discussion	117
5.4.1	<i>Antimicrobial activity against spoilage and potential pathogenic microorganisms</i>	117
5.4.2	<i>Inoculation of vacuum-packaged meat slices</i>	119
5.4.3	<i>Physical and chemical analyses</i>	122
5.5	Conclusions	123
5.6	References	123

6.	Quality and hygiene of beef burgers in relation to the addition of sodium ascorbate, sodium citrate and sodium acetate	127
6.1	Abstract	129
6.2	Introduction	129
6.3	Materials and methods	131
	6.3.1 <i>Sample preparation and experimental design</i>	131
	6.3.2 <i>Microbiological analyses</i>	132
	6.3.3 <i>Physical and chemical analyses</i>	132
	6.3.4 <i>Statistical analyses</i>	133
6.4	Results	133
	6.4.1 <i>Microbiological analyses</i>	133
	6.4.2 <i>Physical and chemical analyses</i>	136
6.5	Discussion	138
6.6	Conclusions	139
6.7	References	140
7.	General discussion	145
8.	Appendix	149
8.1	Screening of species-specific lactic acid bacteria for veal calves multi-strain probiotic adjuncts	151
8.2	Effects of a species-specific probiotic formulation on multiresistant <i>Escherichia coli</i> isolates from the gut of veal calves	152
8.3	Effect of dietary species-specific probiotic in newborn female calves during the first month of life on intestinal Lactobacilli and <i>Escherichia coli</i> populations, health status, and cell mediated immune response	153
9.	Summary	155
10.	Acknowledgements	159

CHAPTER 1

Foreword

1.0 Microbial ecology of food products

Pasteur laid the fundamentals for microbial ecology in 1857 when, studying the effect of a mixed inoculum in a liquid medium, detected that not only the inoculum affected the growth of microorganisms, but also the nature and the characteristics of the medium had an influence. In other words, not only the “seed” but also the “soil” had an impact on microbial growth (Mossel et al., 1995). Further studies by Winogradsky in 1887, were carried on microbial growth in natural habitats, showing that a site characterized by a combination of conditions (“habitat” or “ecological niche”) is colonized by a group of bacteria able to adapt to it. The ubiquitous distribution of bacteria determined the creation of similar ecological niches, which tended to be colonized by similar groups of microbes.

Foods are complex, often nutrient-rich materials, generally able to support a varied microbial population. Food ecosystems are very often heterogeneous and not static, as they change during the time. The ability of each single group of microorganisms to survive and duplicate in a specific food product in dynamic conditions will determine the food microflora (Cocolin and Comi, 2007).

Various microorganisms or the same microorganism at different time, deal with the different situations faced in food products to different loads and in a variety of ways. Communities of microorganisms are static neither in space nor in time: changes in communities of heterotrophic microorganisms cannot result in a steady community, since in absence of continual enrichment there is a progressive depletion of resources.

Food products could be considered as non-steady-state ecosystems, where microorganisms are influenced not only by their different physiological status, by the product characteristics and the storage conditions, but also by their reciprocal interactions.

According to Odum (1953) and Rayner & Webber (1984) the effect of one population on another, under equilibrium conditions, can be detrimental, neutral or beneficial.

Thus, when two populations interact, there are several possible ways in which they may be influenced and different types of outcome of interaction. Interactions usually occur among numerous species, and often simultaneously (Boddy and Wimpenny, 1992).

1.1 Food spoilage

Food, at each stage of the shelf-life should retain its desired sensory, chemical, physical, functional and microbiological characteristics. No food product is able to maintain the original and optimal quality characteristics indefinitely: the time

during which the product remains stable and retains the desired quality is called shelf-life.

Spoilage can be described as a loss of qualitative properties in foods leading to a product which becomes undesirable or unacceptable for human consumption. In few words, spoilage represents any change in food products that determines unacceptability for the consumer from a sensory point of view.

The manifestations of food spoilage are many and varied, and may be visual (discolouration, slime production, colony formation, breakdown of structure, blowing of container) or apparent by smell (off-odour) or taste (off-flavour, increase in acidity).

Even if precise figures of the total economic losses due to food spoilage are unknown, it is clear that it constitutes a huge economical problem. It has been estimated that a quarter of the world's fresh food supply is lost through microbial activity alone (Huis in't Veld, 1996). Fresh produce and fluid milk are counted for nearly 20% of the whole loss while lower percentages are accounted for grain products (15.2%), caloric sweeteners (12.4%), processed fruits and vegetables (8.6%), meat, poultry and fish (8.5%), and fat and oils (7.1%) (Kantor et al., 1997; Doyle, 2007).

Food spoilage may be caused by microbiological, chemical or physical mechanisms. In less developed countries food spoilage due to rodents and other animals is of major concern, while in developed countries, microorganisms are by far the most common cause of spoilage, mainly represented by psychrotrophic bacteria, yeasts and moulds.

The keepability of a food product can be limited by the presence and the growth of human pathogenic bacteria to critical concentration levels above which they become infectious or able to produce toxic concentrations of specific metabolites ("Safe shelf-life"), by the growth of microorganisms to high concentration where they produce metabolites (amines, sulphides, alcohols, aldehydes, ketones and organic acids) resulting in unpleasant off-odour and off-flavour development ("Shelf-life") and by sensory spoilage due to chemical or by physical changes (oxidation, rancidity, colour changes) (Dalgaard, 2009) (Figure 1).

The range of spoilage microorganisms is wide: almost all the groups of microorganisms could contribute to food spoilage depending on several growth factors.

Bacteria are responsible for some of the most rapid and evident spoilage of proteinaceous foods such as meat, poultry, fish, shellfish, milk and some dairy products (Huis in't Veld, 1996).

The growth and activity of spoilage microorganisms is mostly described as a function of substrate base and of chemical and physical parameters such as temperature, pH, aw and atmosphere (Table 1). The importance of these

conditions for the selection of the spoilage microflora cannot be underestimated even if interaction behaviour between microorganisms needs to be considered as well (Gram et al., 2002).

Microbial food safety and food spoilage are often separated even if in the eyes of consumer there is not a clear distinction. Although this separation of causes in food spoilage is convenient, there is really a continuum of causes and effects. Therefore, one of the main problems is to find a relationship between the microbial composition and the presence of metabolites produced by bacterial activity, for the evaluation of spoilage (Borch and Agerhem, 1992; Drosinos and Board, 1994). A combined description of the interaction between microflora developing in a food product and the chemical changes in the same represents an important challenge. A combined approach would be beneficial and useful in relation to the increasing interest in natural preservation systems such as antimicrobial agents.

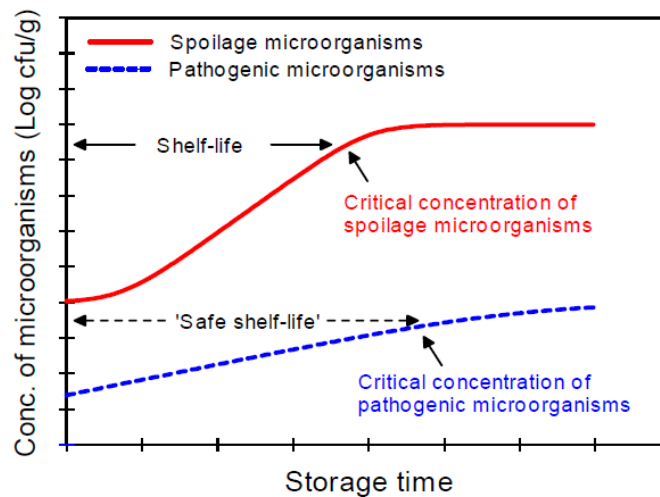


Figure 1: “Shelf-life” and “Safe shelf-life” (Dalgaard, 2009).

Microorganism	Min pH of growth	Min a _w of growth	Max salt tolerance	Min growth T (°C)	Max growth T (°C)
Pathogens					
<i>Salmonella</i> spp.	3.8	0.94	<3-4%	5.2-7 ^D	49.5
<i>Staphylococcus aureus</i>	>4.3	0.85	10-20 %	7	48
<i>Bacillus cereus</i> mesophilic strains	>4.8	0.92-0.93	<5%	15	55
<i>Bacillus cereus</i> psychrotrophic strains	>4.8	0.92-0.93	<5%	4-5	<30-35
<i>Campylobacter</i> spp.	4.0-5.5	0.97	<2%	32	45
<i>Clostridium botulinum</i>	5.0	0.9353	3-12% ^A	15	50
<i>Clostridium perfringens</i>	5.5-5.8	0.97	<5-8% ^B	12	50
<i>Listeria monocytogenes</i>	4.39	0.90-0.92	10%	0	45
<i>Escherichia coli</i>	>5.4	0.96	6%	7	46
<i>E. coli</i> O157:H7	>4.5 ^C	0.96	6.5%	8	44-45
<i>Yersinia enterocolitica</i>	4.2	0.975	^G	-1.3	42
<i>Vibrio parahaemolyticus</i>	4.8	0.94	<10%	5	43
<i>Vibrio cholerae</i>	5.0	0.97	<4%	10	43
Spoilage					
<i>Pseudomonas</i> spp.	4.5-5.3	0.95-0.97	<2.5%	0-7	37-41
Lactic Acid Bacteria	2.8 ^H	0.95	6.5-10% ^H	5-6	50-55
<i>Brochothrix thermosphacta</i>	4.65	-	10%	0	30-37
<i>Shewanella putrefaciens</i>	5.0-5.3	0.95-0.97	-	0-7	37
<i>Photobacterium phosphoreum</i>	5.0	-	-	4	30
Moulds					
<i>Penicillium</i> spp. ^F	<2-2.2	0.79-0.92	-	0,<2,<5,<7,10	>37-40
<i>Aspergillus</i> spp.	3.1	0.76-0.80	10-20%	9	35-40
<i>Fusarium</i> spp. ^E	<2.4 -<3.3	0.90-0.92	-	-2-2.5/5	37

Table 1: Main characteristics of the principal spoilage and pathogenic bacteria and moulds causing microbial deterioration in food products.

Based on Bergey's manual (1984), ICMSF (2005), de W. Blackburn (2006).

- A Growth parameters could change according to the most important groups I (proteolytic) or II (non-proteolytic, saccharolytic), or belonging to group III or IV:
 I: optimal growth 35-40°C, slow at 15°C, not detected below 10-12°C; inhibited by NaCl 10-12%
 II: optimal growth 28-30°C, inhibited by NaCl 5%
 III: inhibited by NaCl 3%
 IV: inhibited by NaCl >3%
- B *Clostridium perfringens* 'optimum % of NaCl is 5%, some strains vary up to 8%
- C *E. coli* O157:H7 can grow at pH 4.5 in a medium adjusted with HCl but not with lactic acid
- D Most strains of *Salmonella* stop growing at 7°C
- E Values are dependant on the species (*F. equiseti*, *F. graminearum*, *F. moniliforme*, *F. sporotrichioides*)
- F Values are dependant on the species (*P. citreonigrum*, *P. citrinum*, *P. crustosum*, *P. verrucosum*)
- G Growth at 5%, not at 7%
- H Most LAB prefer an initial pH of 6-7 to grow, however *Lactobacillus acetotolerans* is able to grow at pH=2.8
 As well for salt tolerance because LAB include halophiles able to grow up to 6.5% (*Enterococci*, some *Pediococci*, *Lactobacilli* and *Leuconostocs*). Some *Weisella* in particular can grow up to 10% of NaCl.

1.2 Microbial interactions

The factors that affect microbial spoilage of foods have been categorized into intrinsic parameters, extrinsic parameters, modes of processing and preservation. Intrinsic parameters are the physical, chemical and structural properties of the food itself (water activity/salt, pH, redox potential, available nutrients, natural antimicrobial substances, smoke components), while the extrinsic parameters are the environmental/storage conditions (temperature, humidity and atmosphere composition) in which the food is maintained.

The growth in food is even dependent on the processing and preservation techniques (heat, high pressure, freezing) that often result in changes in the characteristics of food, determining the associated microflora.

Microbial spoilage is also strictly related to implicit parameters (Mossel et al., 1995) as the “mutual influences among the primary selection of microorganisms resulting from the effect of the above-mentioned parameters” (Huis in’t Veld, 1996).

Implicit parameters are the result of the development of a microorganism, which may have a synergistic or antagonistic effect on the microbial activity of other microorganisms present in the food product (Mossel et al., 1995). Microbial food spoilage is a process involving the growth of microorganisms to loads of 10^7 - 10^9 CFU/g at which microorganisms are supposed to interact and influence the growth of one-another (Boddy and Wimpenny, 1992). As reported above, the interactions could be classified on the basis of the effect in detrimental or beneficial (Fredrickson, 1977).

Several types of interactions are reported in food ecosystems: positive interactions between groups of microorganisms are named “Synergism”: this occurs when one microorganism causes a change in a niche that favours the growth of other species or groups. This effect includes the production or availability of essential nutrients, the changes in pH value, redox potential, and/or in water activity and the elimination of antimicrobial substances, which determines the selective growth of some microbial species.

Changes in environmental conditions, including the competition for essential nutrients, changes in pH value or redox potential or the formation of antimicrobial substances (e.g. bacteriocins) can be also a powerful way for a microorganism to contrast (“antagonism”) other bacterial growth creating a selective benefit (Stiles and Hastings, 1991; Kim, 1993). Several microorganisms express this antagonistic ability exerting a strong influence on food spoilage. The most studied antagonistic microorganisms are Lactic Acid Bacteria, which cause a lowering of pH and may produce antimicrobial compounds (like bacteriocins, organic acids, bacteriocins-like compounds, hydrogen peroxide, etc.). Another example is the spoilage activity of certain Gram negative bacteria, which may produce NH_3 and trimethylamine, that are toxic for other bacteria. Furthermore,

Pseudomonas spp., especially the fluorescent group, produce a range of antibacterial and antifungal compounds like antibiotics and cyanide. Moreover, *Pseudomonas* spp. compete powerfully for iron as they are important producers of siderophores: thanks to this ability, they are used against fungal diseases as biocontrol agents (O'Sullivan and O'Gara, 1992; Ellis et al., 2000).

In the last decades, several observations showed that many microbial interactions in food were limited only to the reduction in the maximum population density, without any significant effect on lag time and growth rate (Buchanan and Bagi, 1997). It was also observed that the growth of the minor population decelerates when the main or the total population count reaches its maximum (Devlieghere et al., 2001; Powell et al., 2004). Based on these proved affirmations, in 2001 Cornu proposed a predictive model including the hypothesis of a simultaneous deceleration of two populations derived from a competition for a common limited resource. Such observations has been already made by Jameson in 1962, who, studying the competitive enrichment for *Salmonella*, affirmed that “when two intestinal organisms, which do not mutually interact by cocillines and bacteriophage, are inoculated together into a liquid medium, each microorganism normally follows at first a growth pattern similar to that which would have followed from a similar inoculum in the same medium in the absence of a competitor. Neither organism normally exhibits its awareness to any appreciable degree, of the other's presence, until the bacterial density of one or other organism has risen to a level near to the molar concentration, when both organisms end their rapid multiplication” (Jameson et al., 1962).

The Jameson effect was later described by Mellefont (2008) as “a race between species to use the resources of the environment to maximize their growth and population numbers: when the resources are nearly finished, the race is over and the growth of each species stops”.

The Jameson effect hypothesis is very simple: both population simultaneously stop growing when the prevalent microbial population between two reaches the maximum load, as discussed by Cornu (2001) and Mellefont et al. (2008): to explain the cases in which the hypothesis of simultaneous deceleration is not applicable, variants of the model were introduced.

In a quantitative risk assessment, investigating the behaviour of *L. monocytogenes* in cold-smoked salmon (FAO-WHO, 2004), it was proposed that the growth of the minority population (*L. monocytogenes*) was partially inhibited by the majority population (LAB) at the reaching of the stationary phase.

The same phenomenon was also detected by Gnanou-Besse et al. (2006): they found a significant effect of LAB population on *L. monocytogenes*, resulting dependent not only on the load of the predominant population, but even on several complex factors such as cell physiological state, background microflora, texture of the product and packaging system which affect the microbial growth.

Moreover, the complexity of these dynamics was underlined even by Buchanan and Bagi (1999) who showed that microbial growth of *L. monocytogenes*, inoculated in co-culture with *Pseudomonas fluorescens* (higher, lower or the same load compared with pathogen monoculture) was affected by temperature, acidity and availability of water in the substrate. Such results indicated that the complexity of microbial community dynamics is better explained if are identified the properties of the product, its microbial composition and inocula loads, the factors affecting microbial interactions and the handling.

The Jameson effect was reported for *Listeria monocytogenes* (Figure 2), *Escherichia coli*, *Staphylococcus aureus*, *Yersinia enterocolitica*, *Bacillus cereus*, *Salmonella* Infantis, *Cronobacter sakazakii* and *Carnobacterium* spp.

Several articles referred to this effect or its variants, including reviews (Powell et al., 2004; EFSA, 2008; Irlinger and Mounier, 2009) and experimental studies concerning:

***Listeria* spp. in:**

- Fishery products (Grau and Vanderlinde, 1992; Duffes et al., 1999; Nilsson et al., 1999; FAO-WHO, 2004; Gimenez and Dalgaard, 2004; Delignette-Muller et al., 2009; Beaufort et al., 2007; Mejiholm and Dalgaard, 2007).
- Poultry and meat products (Coleman et al., 2003; Radin et al., 2007; Lecompte et al., 2008; Ross et al., 2009; Cornu et al., 2011).
- Vegetables (Palmai and Buchanan, 2002; Geysen et al., 2006; Valero et al., 2007; Crépet et al., 2009).
- Microbiological media (Buchanan and Bagi, 1997; Buchanan and Bagi, 1999; Cornu et al., 2002; Mellefont et al., 2008; Antwi et al., 2008; Gnanou-Besse et al., 2010).
- Food processing environment (Guillier et al., 2008).

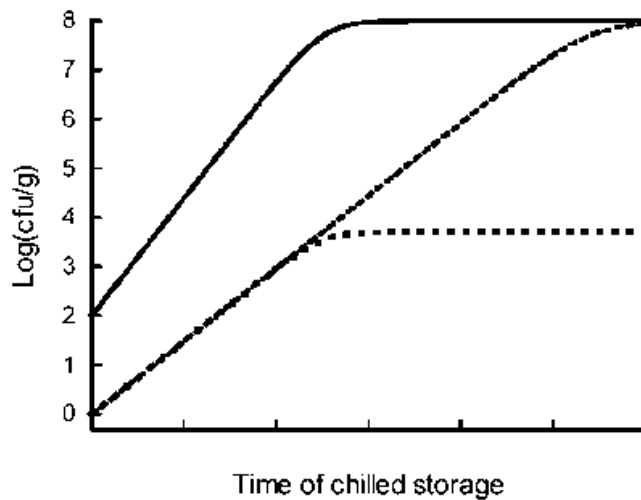


Figure 2: Predicted growth of *Listeria monocytogenes* and lactic acid bacteria (LAB) during chilled storage of cold-smoked salmon. LAB (solid lines), Lm growing alone (dashed line) and Lm growing together with LAB (dotted line) (Dalgaard et al., 2009).

Shiga toxin-producing *Escherichia coli* in:

- Enrichment broth (Vimont et al., 2006).
- Meat products (Coleman et al., 2003; Matagaras et al., 2010)

***Salmonella* spp. in:**

- Poultry products (Coleman et al., 2003; Oscar, 2006).
- Vegetables (Liu and Shaffner, 2007).
- Broth (Komitopoulou et al., 2004).

***Staphylococcus aureus* in:**

- Meat products (Castillejo-Rodríguez et al., 2002).
- Milk (Le Marc et al., 2009).

***Cronobacter sakazakii* in:**

- Enrichment and powdered infant formula (Miled et al., 2010).

1.3 Specific spoilage organisms (SSOs) and fish spoilage

Every seafood produces a specific and characteristic microflora at any given point of the production chain and storage. This microflora is strictly linked to the raw material own bacteria and is a function of the conditions created during processing, preservation and storage.

1.3.1 *Spoilage of fish and fish products*

Fish is one of the most perishable food products. The edible muscle of aquatic animals is rich in proteins and water: in particular, fish tissues contain high level of free non-protein nitrogen (NPN) compounds (free aminoacids, amines, amines oxides, guanidines, nucleotides and their breakdown products, urea and quaternary ammonium salts), easily available for microbial growth. Fish is also rich in free aminoacids, influencing the possible formation of biogenic amines. The carbohydrate content of finfish and crustaceans is negligible, limiting the pH decrease associated with lactic acid production during rigor mortis.

The seafood SSOs produce ammonia, biogenic amines, organic acid and sulphur compounds from aminoacids, hypoxanthine from ATP degradation products and acetate from lactate. Moreover, high concentrations of oxide of trimethylamine (TMAO) are present in some fish muscles. TMAO is an odourless compound, typically reduced to Trimethylamine (TMA) by some bacteria (*Aeromonas* spp., some psychrotolerant *Enterobacteriaceae*, *Photobacterium phosphoreum*, *Shewanella putrefaciens* and *Vibrio* spp.). TMA contributes particularly to the characteristic ammonia-like and fishy off-flavours (ICMSF, 2005). Therefore, fish flesh provides an excellent substrate for microbial growth of most of the heterotrophic bacteria and the related biochemical activities. During handling and storage, quality deterioration of fresh fish rapidly occurs limiting the shelf-life of the product.

1.3.1.1 *Initial microflora*

The initial microbial population of fish reflects the microflora of the environment at the time of capture or harvest, and is subsequently modified by the ability of different microorganisms (mainly bacteria) to multiply in the sub-environments provided by the skin/shell surface, gill areas, and the intestinal content. The muscle tissue and the internal organs are normally sterile: microorganisms can be found on skin, on chitinous shell, on the gills of fish as well as in the intestinal tract (Baross and Liston, 1970; Shewan, 1977). Microbial loads depend on water conditions and temperatures (Table 2). During storage, the microflora changes owing to different abilities of the bacteria to tolerate the preservation conditions.

	Cold water	Warm water
Skin surface	10 ² -10 ⁴ CFU/cm ²	10 ³ -10 ⁶ CFU/cm ²
Gill surface	10 ² -10 ⁴ CFU/cm ²	10 ³ -10 ⁶ CFU/cm ²
Intestinal tract	10 ² -10 ⁸ CFU/g (In molluscs ≥10 ⁶ CFU/g)	10 ² -10 ⁸ CFU/g (In molluscs <10 ³ CFU/g)

Table 2: Initial microbial levels of fish depending on temperatures and water conditions. Based on ICMSF, 2005.

After capture or slaughter and death, finfish is usually stored in ice, favouring a temperature dependant change in microflora composition. Psychrotrophic bacteria normally represent microbial population of fish and shellfish from cold waters, while in fish from temperate waters an increased load of mesophilic bacteria could be observed. Both psychrotrophic and mesophilic microorganisms grow easily at temperatures ranging between 25 and 30 degrees as reported by Gram (1989). The microorganisms present are mainly halotolerant rather than strictly halophilic, revealing an optimal growth at sodium chloride concentrations of 1-3%: this is due to the use of the ice to maintain the products, which determines a decrease of salinity during storage, favouring the survival and growth of halotolerant species. One example of the effect of the salinity in selecting microflora population is in the intestinal tract where halotolerant *Vibrio* spp. are often reported as dominant marine species; Lactic Acid Bacteria are also frequently isolated from fish intestine.

Microorganisms isolated from skin and gills are typically aerobic, in particular *Vibrio* spp. In warm water fishes, the predominant microflora is represented by a higher proportion of Gram-positive cocci (*Micrococci*) and *Bacillus* spp., but even by Gram negative bacteria. The microflora of temperate waters includes genera like *Psychrobacter*, *Moraxella*, *Pseudomonas*, *Acinetobacter*, *Shewanella*, *Flavobacterium*, *Vibrio*, *Aeromonas* and *Cytophaga* but also *Micrococcus* and *Corynebacterium*. *Psychrobacter*-*Acinetobacter*-*Corynebacterium* and *Micrococcus* dominate crustaceans' microflora while *Vibrio* is the main genus isolated from molluscs.

Listeria monocytogenes occurs generally in the environment and especially could be isolated from fish caught or culture closed to land with agricultural run-off (Huss, 1995). It is often isolated from ready to eat seafoods like cold-smoked salmon (Ben Embarek, 1994; Bernardi et al., 2011).

1.3.1.2 Spoilage of fish products

The accumulated metabolic products of microorganisms are the first cause for organoleptic spoilage of raw fish. After, endogenous biochemical changes occur reducing the “fresh fish flavour”.

However, these changes are not implicated in the production of the fishy, sulphide and ammonia typical flavour and of the slimy and pulpy texture of deteriorated fish: these changes are due to microbial action expressed as the oxidative deamination of aminoacids and peptides to ammonia, the reduction of TMAO to TMA, the breakdown of aminoacids containing sulphur and the degradation of nucleotides (ATP-related compounds) (Table 3) (Gram and Huss, 1996; Gram, 1992).

Substrate	Production of spoilage compounds					
	TMAO	Cysteine	Methionine	Other aminoacids	IMP inosine	Carbohydrates lactate
Compound	TMA	H ₂ S	CH ₃ SH	Ketones, esters, aldehydes, NH ₃	Hypoxanthine	Acids
<i>S. putrefaciens</i>	+	+	+	?	+	+
<i>Pseudomonas spp.</i>	-	-	+	+	+	?
<i>P. phosphoreum</i>	+	-	-	?	+	?
<i>Vibrionaceae</i>	+	+	?	?	?	?
<i>Enterobacteriaceae</i>	+	(+)	?	+	+	+
<i>LAB</i>	+	(+)	?	+	?	+
<i>Yeasts</i>	-	-	-	+	?	+
<i>Anaerobic Roads</i>	-		?	+	?	?

Table 3: Substrate and typical compounds produced by bacteria during storage of fresh and packed fish (Gram and Huss, 1996).

The off-odour and off-flavour developed in fish stored in air depend on the fish species.

The spoilage of marine temperate water fish is sensorially characterized by development of offensive fishy, rotten, H₂S-off-odors and off-flavours: in particular, TMAO is primarily associated with some marine fish species and not usually occurs in freshwater fish.

This sensory impression is distinctly different from freshwater fish, where fruity, sulphhydryl off-odours and flavours are more typical (Lima Dos Santos, 1978; Gram, 1989, Gram and Huss, 1996).

The bacteria most commonly identified as spoilage of fresh, iced fish are *Shewanella spp.* and *Pseudomonas spp.* (Liston, 1980; Jørgensen and Huss, 1989),

with *Shewanella putrefaciens* dominating in marine fish stored at 0-2°C (Gram, 1992). Packing of fish in modified atmosphere (including CO₂) inhibit the growth of *Shewanella* spp. and *Pseudomonas* spp. but not the growth of *Photobacterium phosphoreum*, which produces per cell 30 times the amount of tryptamine produced by *Shewanella* spp. (Dalgaard, 1995). Often in CO₂ packed fish Lactic Acid Bacteria dominates the microflora (Emborg et al., 2002). The growth of specific microorganisms during storage depends on several factors: the physical-chemical properties of seafood (e.g. moisture content, pH, presence of preservatives), the processing method applied in the production of the product, the external environment of the food (gas composition) and the storage temperature.

In table 4 are reported some examples of seafood products and related spoilage microorganisms and pathogenic bacteria, previously isolated or able to growth at specific conditions of storage.

Seafood product	Spoilage microorganisms	Pathogens
Frozen raw seafood	<ul style="list-style-type: none"> -10/-5°C: very slow mould and yeasts growth -10/-12°C: no growth 	<i>Listeria monocytogenes</i> , <i>Salmonella</i> spp., <i>Vibrio</i> spp. and <i>Staphylococcus aureus</i> survive well
Minced fish and surimi	<ul style="list-style-type: none"> Spore forming bacteria 	No food-borne illness recorded
Cooked crustacean (frozen and chilled)	<ul style="list-style-type: none"> Gram positive and Gram negative bacteria (<i>Pseudomonas</i> spp. and <i>Acinetobacter-Moraxella</i> spp.) Yeasts 	<i>Vibrio parahaemolyticus</i> , <i>Salmonella</i> spp., <i>Shigella</i> spp., <i>S. aureus</i>
Lightly preserved seafood	<ul style="list-style-type: none"> Gram negatives bacteria, <i>Enterobacteriaceae</i>, <i>P. phosphoreum</i> Lactic Acid Bacteria (some tyramine producer), <i>Bacillus</i> spp. 	Spore-forming Clostridia, <i>L. monocytogenes</i> ,
Semi-preserved fish products	<ul style="list-style-type: none"> Yeasts Lactic Acid Bacteria Anaerobic spore formers 	<i>Clostridium botulinum</i> mesophilic strains
Fermented fish	<ul style="list-style-type: none"> Lactic Acid Bacteria Yeasts 	<i>Clostridium botulinum</i>
Fully dried or salted products	<ul style="list-style-type: none"> Fungi 	<i>Salmonella</i> spp.
Pasteurized products	<ul style="list-style-type: none"> Spore forming Gram positive bacteria, <i>Aeromonas hydrophila</i> 	<i>C. botulinum</i> , <i>L. monocytogenes</i>

Table 4: Principal spoilage and pathogenic microorganisms implicated in seafood spoilage in several fish products. Based on Johnson and Liston (1973), Knøchel and Huss (1984), Wheeler et al. (1986, 1988), Ingham and Moody (1990), Gobat and Jemmi (1993), Ben Embareck (1994), Truelstrup Hansen et al. (1998), Jørgensen et al. (2000).

1.3.2 Specific Spoilage Organisms (SSOs)

Only a part of the fish spoilage microflora participates to the spoilage process: the SSOs are typically present in low number and constitute a small fraction of the total microflora (Gram and Dalgaard, 2002).

The identification of SSOs is determined by the comparison of the sensory and chemical characteristics of spoiled products with those isolated from the spoilage microflora. The ability to produce off-odours and spoilage microorganisms are at the base for the identification of a SSO.

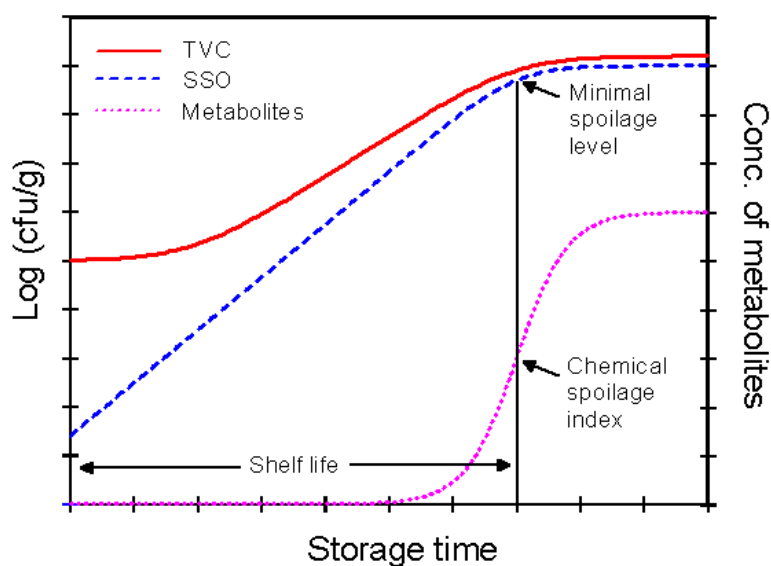


Figure 3: General structure of microbial spoilage in relation to specific spoilage organisms (Dalgaard et al., 1993).

The spoilage potential of a microorganism is the ability of a pure culture to produce the metabolites associated with the spoilage of a particular product (Gram et al., 2002). The spoilage activity of a microorganism determines the production of metabolic compounds (Dalgaard et al., 1993; Dalgaard, 1995). Anyway, the qualitative ability to produce off-odours (spoilage potential) and the quantitative ability to produce spoilage metabolites (spoilage activity) are essential in the identification of a SSO (Gram and Dalgaard, 2002).

The cell concentration of SSO could be called the “minimal spoilage level” and the concentration of the metabolite that corresponds to spoilage can be used as an objective chemical spoilage index (CSI) (Figure 3) (Dalgaard et al., 1993).

Different SSOs could be found in different seafoods and may be a single species. SSO are different depending on the typology of fish and the conditions of

storage (Table 5). For example, *Shewanella putrefaciens* is the specific spoilage bacteria of marine temperate water-fish stored aerobically in ice, while *Pseudomonas* spp. are the specific spoilers of iced stored tropical freshwater fish and together with *S. putrefaciens* the spoilers of marine tropical fish stored in ice (Gillespie and MacRae, 1975; Gram, 1992).

Specific spoilage microorganisms of fresh and chilled fish				
Temperate waters			Tropical waters	
	Marine Fish	Fresh Fish	Marine Fish	Fresh Fish
Aerobic	<i>Shewanella putrefaciens</i> , <i>Pseudomonas</i> spp.	<i>Pseudomonas</i> spp.	<i>Shewanella putrefaciens</i> , <i>Pseudomonas</i> spp.	<i>Pseudomonas</i> spp.
Vacuum	<i>Shewanella putrefaciens</i> , <i>Photobacterium phosphoreum</i>	Gram-positive bacteria, Lactic Acid Bacteria	Lactic Acid Bacteria	Lactic Acid Bacteria
CO₂	<i>Photobacterium phosphoreum</i>	Lactic Acid Bacteria	Lactic Acid Bacteria, TMAO reducing bacteria	Lactic Acid Bacteria, TMAO reducing bacteria

Table 5: Specific spoilage bacteria of fresh and packed fish stored at chilling condition (<4°C) or in ice (Gram and Huss, 1996).

1.4 Ephemeral spoilage organisms (ESOs) and meat spoilage

Meat and meat products are a good support for bacterial growth thanks to its composition characterized by 75% of water and are important sources for protein, fat, essential amino acids, minerals, vitamins and other nutrients (Lawrie, 1985).

1.4.1 Spoilage of meat and meat products

The shelf-life of meat and meat products is the storage time until spoilage: the point of spoilage may be defined by a certain maximum acceptable bacterial level, or an unacceptable off-odour and off-flavour or appearance.

Meat shelf-life depends on the load and the genera of microorganisms initially present and their subsequent growth. Microbial contamination of carcasses is the consequence of the slaughtering process applied. Processing influences not only the quantity of microorganisms, but also the genera of microorganisms present.

After slaughter, if carcasses are cooled properly, bacterial population is $<10^4$ CFU/g, carcasses stored for a week after slaughter could reach 10^6 CFU/g; during cutting and boning the microbial load usually is between 10^4 and 10^6 CFU/g (Sheridan and Lynch, 1992).

The starting mesophilic bacterial load on meat and on cooked meat products is about 10^2 - 10^3 CFU/g or cm^2 (Jackson et al., 1992). After slaughter, under chilling conditions, mesophiles microorganisms will not grow and psychrotrophic bacteria could eventually cause spoilage of carcasses. The rate of spoilage increases with the number of psychrotrophs on the surface of the carcass, with the storage temperature and increases in the a_w of the surface tissue. When the population reaches about 10^7 CFU/ cm^2 , off-odours could be detected, while slime is apparent only when a_w is near 0.99 and the population is about 10^8 CFU/ cm^2 . Spoilage occurs first on moist areas of the carcass (abdominal cavity, cut muscle of the neck, in folds between the fore-leg). Usually only 10% of the bacteria present on meats since the beginning are able to grow at chilling conditions and the fraction (ESOs) causing spoilage is even lower. The spoilage microflora is dominated by psychrotrophic aerobic Gram negative microorganisms, mainly *Pseudomonas* spp. (normally representing more than 50% of the whole microflora), *Acinetobacter* spp. and *Psychrobacter* spp. On the surfaces of sheep and pork carcasses are commonly isolated even *B. thermosphacta* and *Enterobacteriaceae*. Anyway, with some exceptions, generally the same bacterial genera can be isolated from beef, pork, sheep and even chicken carcasses (Nychas et al., 2007; 2008).

During meat processing, the contamination by different pathogenic bacteria is also possible and must be carefully considered. The prevalence of *Salmonella* spp. varies widely: the extension of carcass contamination is influenced by the

prevalence and concentration of these microorganisms in the intestinal tract of the animal (especially sheep, pig and beef). Wrong chilling, storage or transport at temperature above 7-8°C can allow the growth.

Generally, a small percentage of cattle carries *E. coli* O157:H7 in the intestinal tract at slaughter; microorganisms are taken during evisceration and skin removal. Ground beef is also recognized as carrier of *E. coli* (Doyle and Schoeni, 1984).

Campylobacter spp. has been found in sheep carcasses, adult cattle carcasses and pork carcasses (Lammerding et al., 1988) even if during chilling there is a significant reduction in the number of viable *Campylobacter*.

Yersinia enterocolitica is easily found on pig carcasses: several studies reported the ability to grow in meat foods kept under chilling conditions (Bredholt et al., 1999).

Listeria monocytogenes can contaminate carcasses through feces, especially from cattle, pig and sheep and from the surfaces in the slaughter and dressing area (Gobat and Jemmi, 1991).

Staphylococcus aureus can contaminate carcasses through the skin, the equipment used and the hands of workers: chilling storage and transport below refrigeration temperature inhibit the growth of this microorganism. The load of *S. aureus* needs to reach 10³-10⁶ CFU/g for producing sufficient enterotoxin to cause food poisoning (Devriese, 1990; Balaban and Rasooly, 2000).

The incidence of *Clostridium botulinum* in lamb and pork is very low but most of the cases of meat-borne botulism are related to products that are improperly preserved, home-produced or consumed without cooking (Lücke and Roberts, 1993). Anyway, most of the clostridia that occur in raw meats are harmless putrefactive.

The meat industry offers many raw and cooked products prepared in small portions, such as meat slices or comminuted meat.

Operations like cutting, slicing and packaging can lead to contamination by a variety of microorganisms present on tools, handlers and mechanical equipment. In table 6 are reported the main typology of marketed meat and the related spoilage and pathogenic bacteria.

Meat product	Species	Spoilage microorganisms	Pathogens
Frozen raw meat	Beef, veal, lamb, other ruminants	<ul style="list-style-type: none"> -10/-5°C: very slow mould growth (black spots) -10/-12°C: no growth 	<i>Salmonella</i> spp., <i>E. coli</i> , <i>C. jejuni</i> , <i>S. aureus</i> , <i>C. perfringens</i>
	Pork	<ul style="list-style-type: none"> -10/-5°C: very slow mould growth -10/-12°C: no growth 	<i>Salmonella</i> spp., <i>C. coli</i> , <i>Y. enterocolitica</i> , <i>S. aureus</i> , <i>C. perfringens</i>
Minced raw meat (species alone or as a mixture)	Beef, veal, lamb, other ruminants	<ul style="list-style-type: none"> Gram negative bacteria <i>B. thermosphacta</i> LAB 	<i>Salmonella</i> spp., <i>E. coli</i> VTEC
	Pork	<ul style="list-style-type: none"> Psychrotrophic <i>Enterobacteriaceae</i> 	<i>Salmonella</i> spp., <i>C. coli</i> , <i>Y. enterocolitica</i>
Raw cured shelf-stable meat (raw hams, low-acid and high-acid dry sausage)	(Beef, veal, lamb, other ruminants)	<ul style="list-style-type: none"> <i>Enterobacteriaceae</i> <i>Clostridium</i> spp. Moulds 	<i>Clostridium botulinum</i> , <i>Salmonella</i> spp., <i>S. aureus</i> , <i>E. coli</i> VTEC (<i>L. monocytogenes</i> in Chinese sausage)
	Pork	<ul style="list-style-type: none"> LAB 	
Dried meat (charqui, Rou Gan, biltong)	Beef, veal, lamb, other ruminants	<ul style="list-style-type: none"> Molds and Yeasts may grow ($a_w > 0.7$) 	<i>Clostridium botulinum</i> , <i>Salmonella</i> spp., <i>E. coli</i> VTEC, <i>S. aureus</i>
	Pork		<i>L. monocytogenes</i> , <i>B. cereus</i>
Cooked perishable uncured meat	Beef, veal, lamb, other ruminants	<ul style="list-style-type: none"> Gram negative psychrotrophic bacteria (mainly <i>Pseudomonas</i> spp.) <i>Enterobacteriaceae</i> LAB 	<i>Clostridium perfringens</i> , <i>Salmonella</i> spp., <i>E. coli</i> VTEC, <i>S. aureus</i>
	Pork	<ul style="list-style-type: none"> <i>B. thermosphacta</i> 	<i>L. monocytogenes</i>
Fully retorted shelf-stable uncured meat (soups)	Beef, veal, lamb, other ruminants Pork	The heat treatment kills all bacteria present	<i>Clostridium botulinum</i> , <i>Salmonella</i> spp., <i>S. aureus</i>
Cooked perishable cured meats (pate, bacon, nitrites)	Beef, veal, lamb, other ruminants	<ul style="list-style-type: none"> <i>C. putrefaciens</i> Heat resistant psychrotrophic bacteria (<i>Lb. viridescens</i>) 	<i>Salmonella</i> spp., <i>E. coli</i> VTEC, <i>S. aureus</i> , <i>L. monocytogenes</i>
	Pork	<ul style="list-style-type: none"> <i>B. thermosphacta</i> 	<i>Salmonella</i> spp., <i>S. aureus</i> , <i>L. monocytogenes</i>
Shelf-stable cooked cured meats	Beef, veal, lamb, other ruminants Pork	<ul style="list-style-type: none"> <i>Bacillus</i> spp. 	<i>C. botulinum</i> , <i>Salmonella</i> spp., <i>E. coli</i> VTEC, <i>S. aureus</i>

Table 6: Principal spoilage and pathogenic microorganisms implicated in meat spoilage in several meat products. Based on Roberts et al. (1975), Chyr et al. (1981), Bell and De Lacy (1983), Bacus (1984), Hauschild and Simonsen (1986), Cowden et al. (1989), Kramer and Gilbert (1989), Andersen et al. (1991), Glass et al. (1992), Lücke and Roberts (1993), Lücke (2000), Fantelli and Stephan (2001), Brynestad and Granum (2002).

1.4.2 Ephemeral spoilage microorganisms (ESOs)

Spoilage of meat is considered as an ecological phenomenon that includes the changes of the available substrata during the growth of bacteria. Many studies, particularly in meat and meat products, established that spoilage of meat depends on the little fraction of the starting microflora that dominates the product. The prevalence of a particular microbial population depends on several factors that act during processing, transportation and storage. All the environmental determinants constitute a virtual ecological niche in which organisms change in the space and in the time (Boddy and Wimpenny, 1992).

These factors influence the establishment of the particular microbial association and determine the rate of attainment of a climax population called “Ephemeral/specific spoilage microorganisms-ESOs”. These microbial populations are able to adopt various ecological strategies that are the consequence of environmental determinants (stresses, destructive or enrichment disturbance of the ecosystem etc.) and allow them to colonize all the niches (Koutsumanis and Nychas, 2000; Nychas et al., 2007; 2008). Considering raw meat, one of the most known factors affecting microbial growth and consequently the composition of microbial population is the atmosphere, as reported in table 7.

Gas composition	Meat and poultry
Air	<i>Pseudomonas</i> spp.
50% CO ₂ with O ₂	<i>Brochothrix thermosphacta</i>
50% CO ₂	<i>Enterobacteriaceae</i> , Lactic Acid Bacteria
<50% CO ₂ with O ₂	<i>Brochothrix thermosphacta</i> , Lactic acid Bacteria
100% CO ₂	Lactic Acid Bacteria
Vacuum packaged	<i>Pseudomonas</i> spp., <i>Brochothrix thermosphacta</i> , <i>S. putrefaciens</i>

Table 7: Spoilage microorganisms dominating the microbial population of fresh meat stored at 0-4°C under different gas atmospheres. Based on Labadie et al. (1999), Nychas et al. (2007; 2008) and Koutsumanis et al. (2007).

In aerobiosis, the dominating species (ephemeral spoilage) that control the meat spoilage are *Pseudomonas* spp., mainly *Ps. fragi*, *Ps. fluorescens* and *Ps. lundensis* (Table 8 and 9). The reaching of the level of 10⁷-10⁸ CFU/g by the dominating population of *Pseudomonas* spp. determines the production of slime and off-odours (Table 9). Cold-tolerant *Enterobacteriaceae* (in particular *Hafnia halvei*, *Serratia liquefaciens* and *Enterobacter agglomerans*) also occur on chilled meat stored aerobically (Table 7) (Nychas et al., 1998), but in terms of numbers they do not

contribute to the microbial association determining spoilage. Except for lamb, *Brochothrix thermosphacta* and Lactic Acid Bacteria have been detected in the aerobic spoilage flora of chilled meat but they are not considered important in producing spoilage (Holzapfel et al., 1998).

	Growth of spoilage microorganisms					
	Storage	Expected shelf life	<i>Pseudomonas</i> spp.	<i>Enterobacteriaceae</i>	LAB	<i>B. thermosphacta</i>
Meat, normal pH	Air	Days	+++	++	++	++/+++
	Vacuum	Weeks/Months	+	+ /+++	+++	+++
	MAP (<i>high O₂</i>)	Days	+++	++/+++	++/+++	+++
	100% CO ₂	Months	+	+ /+++	+++	+
Meat, High pH	Vacuum	Days	+	++/+++	+++	++/+++
	100% CO ₂	Weeks/Months	+	+ /+++	+++	+
Meat products	Air	Days	+ /+++	+	++	+++
	Vacuum	Weeks	+	+	+++	++/+++
	100%CO ₂	Weeks	+	+	+++	+

Table 8: Expected shelf-life under refrigerated storage and growth ability of bacterial groups and specific bacteria on meat and meat products.

+++ : dominant part of microflora, ++ : intermediate part of the microflora, + : minor part of microflora. Based on Borch et al. (1996).

In anaerobic conditions, such as vacuum packaged meat, the bacterial population is gradually selected towards a CO₂-tolerant microflora. Vacuum-packaged beef may have a storage life of 10-12 weeks at 0°C until the off-flavour becomes unacceptable (Egan, 1983). The bacterial flora is dominated by lactic acid bacteria, mainly *Carnobacterium* spp., *Lactobacillus* spp. and *Leuconostoc* spp. (Borch and Molin, 1988).

A long shelf-life may be obtained using pure CO₂. The time needed to reach 10⁷ cfu/cm² and off-odour is about 10 days in air, and 40 days in 100% CO₂ for pork stored at 4°C (Blickstad et al., 1981). Pure CO₂ discolours and determines an acidification of meats: for these reasons, is often used in minor percentage.

Shelf-life extension by CO₂ results from an immediate selection, as opposed to a gradual one in a vacuum-pack, of lactic acid bacteria growing at a reduced rate (Greer et al., 1993).

Depending on pH and storage temperature, other bacteria such as *B. thermosphacta* and *Enterobacteriaceae* may grow (McMullen and Stiles, 1993), but in 100% CO₂ homofermentative *Lactobacillus* spp. completely dominated the bacterial flora at 4°C (Erichsen and Molin, 1981).

The most frequent bacterial alterations detected in meat and meat products are reported in table 9.

Defect	Meat product	Bacterial genera
Slime	Raw meats	<i>Pseudomonas, Lactobacillus, Enterococcus, Weissella, Brochothrix,</i>
H₂O₂ greening	Raw meats	<i>Weissella, Leuconostoc, Enterococcus, Lactobacillus</i>
H₂S greening	Vacuum packaged meats	<i>Shewanella</i>
H₂S production	Cured meats	<i>Vibrio, Enterobacteriaceae</i>
Sulphide odour	Vacuum packaged meats	<i>Clostridium, Hafnia</i>
Cabbage odour	Bacon	<i>Providencia</i>
Putrefaction	Ham	<i>Enterobacteriaceae, Proteus</i>
Bone taint	Whole meats	<i>Clostridium, Enterococcus</i>
Souring	Ham	<i>Enterococcus, Micrococcus, Bacillus, Clostridium, Lactic Acid Bacteria</i>

Table 9: Common alterations in raw meat and meat products and causal bacteria. Based on Nychas et al. (2006; 2007; 2008) and Skandamys and Nychas (2002).

1.5 Preservation of fish

Preservation of food has been extremely necessary for human survival since the beginning of mankind. Thanks to the application of drying, salting, heating or fermentation growth of microorganisms can be inhibited. The most common fish preservation techniques are the application of low temperature storage, the control of water activity, the control of autolytic enzymatic spoilage, the use of antioxidants and the biopreservation adding lactic acid bacteria (Ghali et al., 2010).

1.5.1 Low temperature storage

The use of low temperature storage is the oldest method to inhibit microbial growth in seafood products. Fish contains a percentage of water between 60 and 80%: the freezing process converts most of this water into ice (Johnston et al., 1994).

When fish is frozen at -5°C , 70% of water is present in crystallized form. This rate increases when temperature lowers, but at temperatures below -30°C , a proportion of the water in the fish muscle still stays at unfrozen state. The final quality of fish products depends on the starting quality of fish at the moment of freezing as well as on freezing/cold storage temperature and freezing rate distribution (slow/fast). Fast freezing produces better quality of fish products; in any case, this technique does not prevent oxidative spoilage as enzymatic spoilage still works. Moreover, freezing operations cannot ensure the prevention of aminoacids deterioration.

Microbial growth is not possible below -9°C ; however, enzyme present still play an important part in fish spoilage. Moreover, it has to be underlined that also if very low temperatures completely inhibit bacterial development, only negligible falls in viable cell numbers can be obtained by this technique. For example, the survival of *L. monocytogenes* in salmon was proved after over than ten months at -20°C .

More recent is the application of slurry ice, which consists of suspensions of spherical microparticles of ice (0.25-0.50 mm diameter) in sea water (with concentrations in ice from 15% for the whole Norwegian salmon, 30% for the northern gutted cod, up to 40% on fishing vessels in Spain), that can be pumped in tubes. This technique offers a better transfer of the chilling, if compared to all the other cooling systems, with a clear advantage of covering the fish without damage (Cattaneo et al., 2008).

Moreover, slurry ice creates a sort of barrier to air and consequently to oxidation and dehydration. The temperature of the liquid mixture is around -1.5°C . If compared to the traditional method in flakes, the use of slurry ice has advantages as characterized by better performance in cooling, enhancing the shelf-life and

less damaging fish skin, thanks to contact with microscopic particles, and determines a better maintenance of the sensory characteristics of the fish. The extension of the shelf-life derives from the decrease in the microbial growth and the slowdown of the biochemical degradation mechanisms as reported by Yamada et al. (2002). The system resulted to be particularly adapt for small species such as anchovies and sardines which are sold mainly as fresh products or chilled but intended for further processing. Moreover the slurry ice constitutes a good slaughter method (Huidobro et al., 2001).

1.5.2 Controlling water activity

The water activity represents the ratio of the water vapour pressure of food to the water vapour pressure of pure water under the same conditions (CSIRO, 2005). It represents the relative amount of water that is available for tissue or microbial metabolic reactions. Thanks to drying, addition of chemical compounds (mainly sodium chloride and sugars) or combining the drying and the addition, is possible to control of water activity, binding up the free water molecules and creating an osmotic imbalance resulting in cell growth inhibition (Ray, 2004).

1.5.3 Controlling autolytic enzymatic spoilage

As the fish degradation process starts with autolytic activity, it is important to reduce the enzymatic action: this can be done removing the involved enzymes or developing new techniques able to inhibit their activity. Gutting the fish immediately after capture can avoid the invasion of digestive tract by proteases through abdominal cavity to the muscles (Pedrosa-Menabrito and Regenstein, 1988). The addition of sodium chloride showed to inactivate autolytic enzymes in marine species (Reddi et al., 1972; Siringan et al., 2006). The use of organic acids (lactic, acetic, propionic) was also reported to be effective in reduction of enzymes activity (Martinez and Gildberg, 1988; Hidalgo et al., 1999).

1.5.4 Control of oxidative spoilage through phenolic antioxidants and Ethylenediaminetetraacetic Acid (EDTA)

In order to inhibit lipid oxidation, the free radical mechanism catalysts (molecular oxygen and transition metals) need to be removed. The most used oxidation inhibitory additives are phenolic antioxidants and Ethylenediaminetetraacetic Acid (EDTA).

Derivates of phenol (Butylated Hydroxyanisole [BHA], Butylated Hydroxytoluene [BHT] and Tertiary Butylhydroquinolone [TBHQ]) possess antimicrobial properties, especially against Gram negative bacteria, fungi, viruses

and protozoa thanks to the adverse effects on cell membranes and enzymes (Brannen et al., 1980). EDTA is a lipid oxidation inhibitor, known as chelating, sequestering and metal complexing agent: the addition of this acid helps the removal of free radicals in fish. It has also shown antimicrobials properties thanks to binding divalent cations in bacterial cell walls (Shelef and Seiter, 2005). Following the Reg. EU 1333/2008, EDTA is allowed only for canned fish, crustaceans and molluscs and for frozen and deep frozen crustaceans.

1.5.5 Use of antimicrobials

The most used antimicrobials in seafood products are:

- **Organic acids:** the most used compounds are **Lactic acid/Lactates**, **Ascorbic acid** and **Benzoic acid**.

Sallam (2007) evaluated the effects of the addition of sodium acetate, sodium lactate and sodium citrate applied on salmon slices: significant reduction in K value, Hypoxanthine concentration (Hx), Total volatile Base Nitrogen (TVBN) and Trymetilamine (TMA) was recorded. Park et al. (2005) recorded an antimicrobial action of lactic acid against *Salmonella* Enteritidis: at a concentration of 5 and 10%, the pathogen's growth was slowed.

Ascorbic acid, sodium ascorbate and D-isoascorbate show to enhance antimicrobial activity of sulphites and nitrites, thanks to the antioxidant properties as well as the iron-sequestering activity (Tompkin, 2005).

Benzoic acid and sodium benzoate are used in acidic products as growth inhibitor of yeasts and fungi: the combination of this acid with nitrogen starvation conditions is suggested by Hazan et al. (2004) to enforce the effectiveness of preservation from yeasts, due to the intrinsic ability e.g. of *Saccharomyces* to resist to benzoic acid under tolerable toxicological limits.

- **Nitrites:** added as salts, they possess an inhibiting effect against *C. botulinum*, *S. aureus* and *Y. enterocolitica* and are extremely effective in controlling colour and odour modifications and lipid oxidation (Sindelar and Houser, 2009). Nitrites are involved in reactions with enzymes in vegetative cells and germinating spores, restrictions use of iron by bacteria and limiting transport due to interference with membrane permeability. Nitrites' effect is enhanced at low pH (5-6) and in presence of reducing agents like ascorbate (Ray, 2004). Their use is not allowed in EU and Canada but the presence of *C. botulinum* in seafood of Baltic Sea, with a high prevalence of type E, and the increasing trend to diminish the level, suggest to reconsider the use of nitrites in smoked vacuum packed seafood, as in USA.

1.5.6 Biopreservation by Lactic Acid Bacteria

Among alternative seafood preservation strategies, particular attention has been paid to biopreservation techniques, which extend shelf life and improve microbial quality of fish products.

Biopreservation refers to extended storage life and enhanced safety of foods using the natural microflora and (or) their antibacterial products. Lactic acid bacteria have a major potential for use in biopreservation because they are safe to consume and during storage, they naturally dominate the microflora of many foods (Stiles, 1996).

LAB are often naturally present in food products and act as competitors against spoilage and pathogenic bacteria, thanks to the production of a wide range of metabolites like organic acids, diacetyl, acetoin, hydrogen peroxide, reuterin, reutericyclin, antifungal peptides and bacteriocins (Ghambari et al., 2013).

Although LAB are not considered as the natural dominant microflora of aquatic environment, certain genera, including *Carnobacterium*, *Enterococcus*, *Lactobacillus* and *Lactococcus* have been isolated from both lightly preserved and semipreserved fish products deriving from fresh and seawater fish.

Aim of biopreservation of seafood products is to control microbial deterioration without negative impact on sensorial quality. To be effective as seafood biopreservants, LAB do not have to determine a negative impact on consumer health as well as not cause any detrimental effect on food. As some LAB contribute to spoilage or degradation of food, is necessary to consider their effect on quality parameters. Moreover, important requisites for the use as biopreservants is the ability to produce sufficient antimicrobial metabolites able to contrast food-borne pathogens, spoilage bacteria and fungi and the capacity to resist at the adverse conditions during processing and storage (Ghambari et al., 2013).

In table 10 are reported the main protective cultures or the bacteriocins applied to fish and seafood products and their relative effect detected, based on literature data.

Product	Protective culture or bacteriocin applied	Effect	Reference
<i>Fresh fillets</i>			
Catfish	<i>Lc. cremoris</i> ssp. <i>cremoris</i> ATCC 19257	Improved odour and appearance	Kim and Hearnberger, 1994
Catfish	<i>Bif. adolescentis</i> , <i>Bif. infantis</i> , <i>Bif. longum</i>	Extended shelf-life	Kim et al., 1995
Horse mackerel	<i>Pediococcus</i> spp.	Improved sensorial quality	Cosansu et al., 2011
Indian mackerel	<i>Ped. acidilactici</i> , <i>Ped. pentosaceus</i> , <i>Str. thermophilus</i> , <i>Lc. lactis</i> , <i>Lb. plantarum</i> , <i>Lb. acidophilus</i> , <i>Lb. helveticus</i>	Controlled spoilage amines and bacteria	Cosansu et al., 2011
Rainbow trout	Nisin containing solution of <i>Lc. lactis</i> ssp. <i>Lactis</i> NCFB 497	No effect	Kisla and Ünlütürk, 2004
Salmon	<i>Lb. sakei</i> LAD and <i>Lb. alimentarius</i> BJ33	Improved sensory attributes	Morzel et al., 1997
Tilapia	<i>Lb. casei</i> DSM 120011 and <i>Lb. acidophilus</i> 1M	Improved biochemical quality and microbial aspects	Ibrahim and Sahla, 2009
Tilapia	<i>Lb. casei</i> DSM 120011 and <i>Lb. acidophilus</i>	Extended shelf-life and safety	Daboor and Ibrahim, 2008
Turbot, VP and MAP	Ent-producing enterococci	Anti-listerial, anti-staphylococcal, anti-bacilli	Campos et al., 2012
VP fresh plaice	<i>Bif. bifidus</i>	Inhibition of <i>Pseudomonas</i> spp. and <i>P. phosphoreum</i>	Altieri et al., 2005
VP rainbow trout	<i>Lb. sakei</i> CECT 4808 and <i>Lb. curvatus</i> CECT 904T	Extended shelf-life	Katikou et al., 2007
VP rainbow trout	Sakacin A producing strain of <i>Lb. sakei</i> (Lb706)	Inhibition of <i>L. monocytogenes</i>	Aras and Hüsav, 2005

a)

Product	Protective culture or bacteriocin applied	Effect	Reference
<i>Cold smoked fish</i>			
CO ₂ packed CSS*	Nisin	Reduction of <i>L. monocytogenes</i>	Nillson, 1997; Nillson et al., 1999
CSS	Sakacin P	Inhibition of <i>L. monocytogenes</i>	Aasen et al., 2003
CSS	<i>C. maltaromaticum</i> CS526	Inhibition of <i>L. monocytogenes</i>	Yamazaki et al., 2003
CSS	<i>C. divergens</i> V41, <i>C. divergens</i> V1, <i>C. divergens</i> SF668	Inhibition of <i>L. monocytogenes</i>	Brillet et al., 2005
CSS	<i>Lb. sakei</i>	Inhibition of <i>L. innocua</i>	Weiss and Hammes, 2006
CSS	<i>Lb. casei</i> , <i>Lb. plantarum</i> , <i>C. maltaromaticum</i>	Inhibition of <i>L. innocua</i>	Vescovo et al., 2006
CSS	<i>Lb. casei</i> T3, <i>Lb. plantarum</i> PE2	Inhibition of <i>L. innocua</i>	Vescovo et al., 2006
CSS	<i>E. faecium</i> ET05	Inhibition of <i>L. innocua</i>	Tomè et al., 2005
CSS	<i>C. divergens</i> M35	Inhibition of <i>L. monocytogenes</i>	Tahiri et al., 2009
VP CSS**	<i>Carnobacterium</i> spp.	Improved sensorial characteristics	Leroi et al., 1996
VP CSS	<i>C. piscicola</i> V1, <i>C. divergens</i> V41, Divercin V41	Inhibition of <i>L. monocytogenes</i>	Duffes et al., 1999a,b
VP CSS	Sakacin P-producing <i>Lb. sakei</i> and Sakacin P	Inhibition of <i>L. monocytogenes</i>	Katla et al., 2001
VP CSS	<i>L. sakei</i> 3, 9 and 11	Inhibition of <i>L. monocytogenes</i>	Bernardi et al., 2011
VP Rainbow trout	Nisin	Inhibition of <i>L. monocytogenes</i>	Nykanen et al., 2000
<i>Shrimp</i>			
Brine shrimp	Nisin Z, Carnocin UI49 and crude Bavaricin A	Extended shelf-life	Einarsson and Lauzon, 1995
Chilled shrimp	Nisin	Inhibition of <i>Pseudomonas</i> spp. and H ₂ S producing bacteria	Shirazinejad et al., 2010
Cooked shrimp	<i>Lc. piscium</i> CNCM I-4031	Inhibition of <i>B. thermosphacta</i> and improvement sensorial indices	Fall et al., 2010
Cooked shrimp	<i>C. maltaromaticum</i>	No effect	Laursen et al., 2005
VP cooked shrimp	<i>Lc. piscium</i> EU2241 and <i>Leuconostoc gelidum</i> EU2247	Inhibition of <i>L. monocytogenes</i> and <i>S. aureus</i>	Matamoros et al., 2009

b)

Table 10 a) and b): Survey of literature dealing with biopreservation of fish and fish products. Based on Ghambari et al. (2013)

CSS*: cold smoked salmon

VP**: vacuum packaged

1.6 Meat preservation

Traditionally, meat preservation methods have been grouped into three categories based on control by temperatures, by moisture and by inhibitory processes, although a particular method of preservation may involve several antimicrobial principles. Each control step may be regarded as a “hurdle” against microbial proliferation, and combination of processes (so called Hurdle technology [HT]) can be developed to achieve particular objectives in terms of both microbial and organoleptic qualities (Lawrie and Ledward, 2006; Zhou et al., 2010). Actually, the most investigated new preservation technologies for fresh meat are non-thermal inactivation technologies such as high hydrostatic pressure (HHP), new packaging systems such as modified atmosphere packaging (MAP) and active packaging (AP), natural antimicrobial compounds and biopreservation.

1.6.1 Cold temperatures

Temperature below or above the optimum range for microbial growth will have a preservative action. For fresh meat, refrigeration has been the traditional preservation method. The most used refrigeration methods are:

- **Chilling:** it is critical for meat hygiene, shelf life, appearance and eating quality. Chilling in air reduces carcass surface temperature and enhances carcass drying, both of which reduce the growth of bacteria (Ockerman and Basu, 2004). Rapid carcass chilling increases product yield due to lower evaporation from the surface, and operates for a better bacterial growth.
- **Freezing:** fast freezing produces minute intracellular ice crystals; the rate of freezing is dependent not only on the bulk of the meat but also on temperature of refrigeration environment, on the method applied and, in presence of little pieces of meat, on the nature of wrapping material used. The temperature of -55°C is considered the ideal storage condition for frozen meat in order to prevent quality changes but it is rarely used.
- **Superchilling:** it is a process where a minor part of the product’s water is frozen. After initial surface freezing, the ice distribution equilibrates and the product obtains a uniform temperature at which it is maintained during storage and distribution (Magnussen et al., 2008). At superchilling temperatures, most microbial activity is inhibited or terminated: the ice present in superchilled products protects the meat from temperature rises in poor cold chains even if increase in drip loss could occur. This technique is

able to prolong the shelf life of 1.4-4 times if compared to the traditional chilling (Zhou et al., 2010).

1.6.2 Chemical preservatives

- **Ozone** have been used to discourage the growth of surface microorganisms on beef carcasses during prolonged storage at chill temperatures. Ozone is one of the most efficient oxidative agents; it is active at different concentrations, against bacteria, yeasts and moulds. Even if ozone does not leave toxic residues in meat, its use in a production environment can be dangerous for workers. Moreover, it accelerates the oxidation of fat and is more effective on microorganisms related to air than those on meat.
- **Organic acids** highlighted potential positive benefits in terms of bacterial inhibition and stabilization of sensory characteristics (flavour, colour, juiciness). In particular, lactic acid is frequently effective in meat preservation. Recently the antimicrobial effects of other organic compounds such as citric, acetic and ascorbic acid and their salts have been investigated *in vitro* and in meat products (Harris *et al.*, 2012). The antimicrobial activity of acetic acid and its salts for example is due to their ability to lower the pH and to compromise the bacterial cell walls (Lück and Jager, 1998). Citric acid and citrates are considered as mild antimicrobial agents, and can be used with other organic acids, resulting in an extension of the lag phase of the autochthonous microorganisms of sheep and goat meat, leading to lower total viable counts (Ahmed et al., 2003).

1.6.3 Ionising radiations

Ionising radiations have been studied as a method of direct microbial inhibition for preservation of meat since 1940. FAO and WHO proposed 10 kGy as limit dose that should be accepted for preserving almost all the foodstuffs (FAO/WHO, 1999). The majority of industrial facilities use the Cobalt as radiation source, as it produces a strong gamma ray emission and it is not soluble in water (Ahn and Lee, 2006).

The advantages of ionising radiations for food preservation include their highly efficient inactivation of bacteria, the absence of significant chemical alteration of the product and the efficacy also for foodstuff characterized by high thickness, giving the possibility to treat also packaged meat stored in containers (Lawrie and Ledward, 2006). High dose irradiated raw meat shows colour changes due to the susceptibility of the myoglobin molecule to energy input and alterations in

the chemical environment. Radiation treatment resulted in no significant loss of thiamine content (EFSA, 2011; Graham et al. 1998).

1.6.4 High hydrostatic pressure

High hydrostatic pressure (HHP) is a non thermal technology of primary interest, as it determines a higher stability in foodstuffs. HHP is effective in inactivating microbial population, resulting in a reduction of spoilage and pathogenic microorganisms. Moreover, it can inactivate certain food enzymes at low temperatures without changing the sensorial and nutritional characteristics the products (Patterson et al., 1995; Patterson, 2005). In raw and marinated meats HHP exerts an inhibition activity against *Salmonella* spp. and *Listeria monocytogenes* (Hugas et al., 2002).

HHP combined with moderate temperature showed to change the chemical properties of the meat leading to improved tenderness of meat (Sikes et al., 2010). HHP at low temperature HHP can exert negative effects on fresh meat colour.

1.6.5 Packaging

Packaging is usually applied with the aim of protecting meat products from deteriorative effects (e.g. discolouration, off-flavour, off-odour, nutrient loss, texture changes).

For raw chilled meat, the packaging methods usually applied are:

- **Vacuum-packaging (VP):** the lack of O₂ can minimize the deterioration due to oxidative reactions, and can reduce aerobic bacteria growth, which usually represent the main spoilage population of raw meat.
- **Modified atmosphere packaging (MAP):** MAP is a food-packaging method in which the proportions of carbon dioxide, nitrogen, and oxygen in a sealed container are different from those in the normal (ambient) air with the aim to enhance the foods shelf life. MAP requires packaging materials able to maintain a constant environment during storage. It is readily available and is widely used also if it is the most cost-effective packaging (McMillin, 2008). MAP with high O₂ concentration is generally used for red meat marketing, as it maintain for a longer time the redness typical of fresh meat. Anyway, low concentrations of O₂ are required to inhibit and contrast microbial growth. Low O₂ MAP may be used as a barrier package with an anoxic atmosphere of N₂ and CO₂. CO₂ can be considered a mild antibacterial agent, thanks to its acidifying activity at concentration higher

than 20%, and is usually applied for modified atmosphere packaging of raw meat. CO has also been used in low O₂ retail packaging system: meat can be exposed to CO before packaging or CO may also be used to gas flush VSP packages before sealing (Belcher, 2006). The use of CO is still not allowed for food packaging in EU due to its dangerousness for workers.

- **Active packaging (AP):**

Following the “EU Guidance to the Commission Regulation (EC) 450/2009 on active and intelligent materials and articles intended to come into contact with food”, active packaging is defined as a type of food packaging with an extra function, in addition to that of being a protective barrier against external stimulus. Active packaging is intended to influence the packed food, can absorb chemical compounds from the food or the environment and can also release substances into the food or the environment such as preservatives, antioxidants, flavourings, etc. According with the definition, active materials and articles are grouped in:

Absorbing/scavenging systems, including:

- **Moisture absorbers**: pads used for example to absorb the drip from meat, poultry and fish in presentation packs. They could be made by a laminate of plastic gauze, adhesive and pads containing polymeric fibres or granular polyacrylates alone or in combination with natural cellulose all contributing to the absorbing function of the pad. Materials and articles functioning on the basis of the natural constituents only (e.g. 100% cellulose), are not designed to deliberately incorporate components that would release or absorb substances.
- **Oxygen scavengers**: used often in packaged pasta, milk powder, biscuits, etc. These scavengers are usually in the form of sachets. They capture residual oxygen from inside the packaging (from the environment close to the food or from the foodstuff itself) to reduce contact with oxygen, limiting in that way the microbiological growth, the chemical changes of the food and prolonging its shelf-life of the foodstuffs.

Releasing systems: are packaging that contain substances such as preservatives, antioxidants, flavourings, enzymes. These are intentionally released into the packaged food or in the environment surrounding the food with the aim to maintain or extend the shelf-life.

Systems with substances grafted or immobilized on wall of the packaging: they are packaging materials containing a substance such as an additive or

enzyme, which is grafted on the surface in contact with food and exert a technological effect on it. These materials incorporate active components that influence the condition of the food without intentional migration. This category of packaging is thus similar to the releasing systems with the difference that the active substance is not released into the food but it stays grafted or "immobilized" on the packaging.

Intelligent materials and articles: they are materials and articles which monitor the condition of packaged food or the environment surrounding the food. Intelligent packaging systems give the information on the conditions of the food, but they do not have the intention to release their constituents into the food. The intelligent component may be placed outside the surface of the package and can be separated from the food by a functional barrier. The indication is often a visual signal. A packaging with a time-temperature indicator is an example of an intelligent packaging.

1.6.6 Thermal alternative technologies

Quick thermal technologies such as microwave and radiofrequency tunnels or steam pasteurization bring new possibilities to the decontamination of meat products, and in particular ready-to-eat products. Their application after final packaging is useful to contrast cross-contamination that could happen during post-processing handling.

1.6.7 Biopreservation

In biopreservation of raw meat, storage life is extended thanks to the use of natural or controlled microflora, mainly lactic acid bacteria (LAB) and or their antimicrobial products (bacteriocins, organic acids) (Hugas, 1998).

As already mentioned for fish and fish products, LAB have a long history of safe use in food, and represent the main natural microflora of vacuum packaged raw meat. They can exert their antagonism through competition for depletion of nutrients and/or production of several antimicrobial substances such as organic acids (lactic and acetic acid), carbon dioxide, hydrogen peroxide, diacetyl, ethanol and bacteriocins.

LAB could be included in meat batter, sprayed onto the surface or added through active packaging depending on the type of product (Aymerich et al., 2008).

To be successful in raw meat biopreservation, LAB cultures must survive at refrigeration temperatures, compete with the relative high indigenous microbial

population load of raw meat, actively inhibit pathogenic and specific spoilage bacteria, and not alter the sensory properties of the meat.

Some studies have also revealed the potentially positive activity exercised by LAB enzymes in improving meat flavour, tenderness and nutritional quality during vacuum storage (Fadda et al., 1999).

In table 11 are reported the main protective cultures or the bacteriocins applied to meat and meat products and their relative effect detected, present in literature.

Product	Protective culture or bacteriocin applied	Antagonistic effect against
Spanish fermented sausages	<i>L. lactis</i> BB24-Nisin	<i>Cl. botulinum</i> , <i>Cl. perfringens</i> , <i>L. monocytogenes</i> , <i>S. aureus</i>
Thai fermented sausages	<i>L. lactis</i> WNC20-Nisin Z	Other LAB, <i>B. cereus</i> , <i>Cl. botulinum</i> , <i>E. faecalis/faecium</i> , <i>S. aureus</i>
Spanish fermented sausages	<i>Lb. sakei</i> 148, V18-Lactocin S	Other LAB, <i>Cl. botulinum</i> , <i>Cl. perfringens</i> , <i>E. faecalis/faecium</i>
Norwegian fermented sausages	<i>Lb. sakei</i> L45-Lactocin S	Other LAB, <i>Cl. botulinum</i> , <i>Cl. perfringens</i> , <i>E. faecalis/faecium</i>
Beef	<i>Lb. sakei</i> LTH673, 674-Sakacin K, P	Other LAB, <i>E. faecalis/faecium</i> , <i>L. monocytogenes</i>
Italian fermented sausages	<i>Lb. sakei</i> I151-Sakacin P	<i>L. monocytogenes</i>
Beef, meat products	<i>Lb. sakei</i> Lb706-Sakacin A	Other LAB, <i>E. faecalis/faecium</i> , <i>L. monocytogenes</i>
Spanish fermented sausages	<i>Lb. sakei</i> CTC494-Sakacin K	Other LAB, <i>E. faecalis/faecium</i> , <i>L. monocytogenes</i>
Beef	<i>Lb. sakei</i> MN-Bavaricin MN	<i>L. monocytogenes</i>
Fermented sausages	<i>Lb. brevis</i> SB27-Brevicin 27	Other LAB, <i>B. cereus</i>
German meat products	<i>Lb. curvatus</i> LTH1174-Curvacin A	Other LAB, <i>B. cereus</i> , <i>L. monocytogenes</i>
Argentine fermented sausages	<i>Lb. curvatus</i> CRL705-Lactocin 705	Other LAB, <i>B. cereus</i> , <i>B. thermosphacta</i> , <i>Propionibacterium</i>
Minced beef products	<i>Lb. curvatus</i> FS47-Curvacin FS47	Other LAB, <i>B. cereus</i> , <i>E. faecalis/faecium</i>
Greek fermented sausages	<i>Lb. curvatus</i> L442-Curvacin L442	Other LAB, <i>L. monocytogenes</i>
Spanish fermented sausages	<i>Lb. plantarum</i> CTC305-Plantaricin A	Other LAB, <i>L. monocytogenes</i>
Vacuum-packed meat	<i>Lc. gelidum</i> UAL187-Leucocin A	Other LAB, <i>E. faecalis/faecium</i> , <i>L. monocytogenes</i>
Vacuum-packed meat	<i>Lc. mesenteroides</i> TA33a-Leucocin A	Other LAB, <i>E. faecalis/faecium</i> , <i>L. monocytogenes</i>
Greek fermented sausages	<i>Lc. mesenteroides</i> L124	Other LAB, <i>L. monocytogenes</i>
Greek fermented sausages	<i>Lc. mesenteroides</i> E131	<i>L. monocytogenes</i>
Vacuum-packed meat	<i>Lc. carnosum</i> TA11a-Leucocin A	Other LAB, <i>E. faecalis/faecium</i> , <i>L. monocytogenes</i>
American-style sausages	<i>P. acidilactici</i> PAC1.0-Pediocin PA-1/ach	Other LAB, <i>Cl. botulinum</i> , <i>Cl. perfringens</i> , <i>L. monocytogenes</i> , <i>Propionibacterium</i>

Spanish fermented sausages	<i>P. acidilactici</i> L50-Pediocin L50	Other LAB, <i>Cl. botulinum</i> , <i>Cl. perfringens</i> , <i>E. faecalis</i> , <i>L. monocytogenes</i> , <i>Propionibacterium</i> , <i>S. aureus</i>
Spanish fermented sausages	<i>P. pentosaceus</i> Z102-Pediocin PA-1	Other LAB, <i>Cl. botulinum</i> , <i>Cl. perfringens</i> , <i>L. monocytogenes</i> , <i>S. aureus</i>
Vacuum-packed meat	<i>C. piscicola</i> LV17B-Carnobacteriocin B2	<i>L. monocytogenes</i>
Processed meat	<i>C. piscicola</i> LV17A-Carnobacteriocin A	Other LAB, <i>L. monocytogenes</i>
Spoiled ham	<i>C. piscicola</i> JG126-Piscicolin 126	<i>L. monocytogenes</i>
Vacuum-packed meat	<i>C. piscicola</i> KLV17-Carnobacteriocin B1/B2	Other LAB, <i>E. faecalis/faecium</i> , <i>L. monocytogenes</i>
Vacuum-packed meat	<i>C. divergens</i> 750-Divergicin 750	Other LAB, <i>Cl. perfringens</i> , <i>E. faecalis/faecium</i> , <i>L. monocytogenes</i>
Vacuum-packed meat	<i>C. divergens</i> LV13-Divergicin A	Other LAB
Spanish fermented sausages	<i>E. faecium</i> CTC492-Enterocin A	Other LAB, <i>Cl. perfringens</i> , <i>L. monocytogenes</i>
Spanish fermented sausages	<i>E. faecium</i> CTC492-Enterocin B	<i>L. monocytogenes</i>
Italian fermented sausages	<i>E. casseliflavus</i> IM416K1-Enterocin 416K1	<i>L. monocytogenes</i>
Vacuum-packaged raw beef	<i>Lactobacillus curvatus</i> CRL705-lactocin 705 and 705 AL	<i>Brochothrix thermosphacta</i> and spoilage LAB
Frozen ground-beef patties	<i>Lactobacillus curvatus</i> CRL705 and <i>Lactococcus lactis</i> CRL1109 in combination with Na ₂ EDTA	<i>Escherichia coli</i> O157:H7
Cooked meat products	<i>Leuc. carnosum</i> 4010 bacteriocins or purified	Living protective culture were more effective in preventing growth of <i>L. monocytogenes</i> than the use of the partially purified leucocins 4010 or bacteriocin produced during fermentation
Brazilian meat and meat products	<i>Lactobacillus curvatus</i> , <i>Leuconostoc mesenteroides</i> , <i>Leuconostoc sp.</i>	<i>L. monocytogenes</i>
Chill-stored, vacuum-packaged beef	<i>Leuconostoc gelidum</i> UAL187 <i>Lb. sake</i> 1218	Beef spoilage microorganisms

Table 11: Survey of literature dealing with biopreservation of meatborne LAB. Based on Leisner et al. (1996), De Martinis and Freitas (2003), Jacobsen et al. (2003), Castellano et al. (2004), Castellano and Vignolo (2006), Castellano et al. (2008), Castellano et al. (2010).

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CHAPTER 2

Objectives

2. Objectives

Consumers are increasingly more concerned about safety in food than in any other product, including medicines (Prendergast, 1997) and demand for food that is safe, free from pathogens, with minimal processing but with an unaltered sensorial quality. As a response to these requests, current trends in food industry include the investigation of alternative inhibitors to ensure food safety. In the last century, several alternative or complementary preservation technologies were developed. Different approaches have been studied and consequently there are many promising technologies currently being evaluated in industrial production. All these technologies have the same goal of being mild for food but contrasting the pathogenic and spoilage microorganisms, guaranteeing the natural appearance of the product.

Foods represent complex environments, generally able to support a diversified microbial population. They could be considered as heterogeneous and not static ecosystems: the aim of preservation techniques is to influence this environment by modification of the microbial population or by changing the food conditions in order to settle a more favourable equilibrium.

In this work, different approaches for the conservation of meat and fish products were evaluated, with particular attention to the application of bioprotective cultures and the addition of mild organic salts. The studies performed were focused on the antagonistic activity towards spoilage and potential pathogenic target microorganisms and on the influences of these methods on microbial, chemical-physical and sensorial stability of the products considered.

The application of bioprotective cultures to ensure the hygienic quality is one of the most promising tools (Stiles, 1996). Biopreservation has increased attention as means of naturally controlling the shelf-life and safety of meat and fish products.

On the other hand, the application of organic acids and salts is known to exert potential benefits in terms of bacterial inhibition and stabilization of sensory characteristics which are demanded by consumers and required for retail markets (Lück and Jager, 2002).

2.1 References

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In the following part of the thesis are reported the main studies conducted during the PhD period. In the first study “**Effect of the Lactic Acid Bacteria on the control of listerial activity and shelf-life of smoked salmon scraps**” the potential protective activity of lactic acid bacteria isolated and identified from cold smoked salmon scraps was evaluated towards autochthonous and ATCC *Listeria monocytogenes* strains.

Studies 2 and 3 faced the potential application of different LAB bioprotective mixtures to raw meat and meat preparations.

In the second study “***In vitro* evaluation of *Lactobacillus animalis* SB310, *Lactobacillus paracasei* subsp. *paracasei* SB137 and their mixtures as potential bioprotective agents for raw meat**” *Lactobacillus animalis* SB310 and *Lactobacillus paracasei* subsp. *paracasei* SB137 were investigated in terms of *in vitro* antimicrobial activity towards spoilage and potential pathogenic microorganisms for a possible application as bioprotective agents in vacuum-packed raw meat. These two strains were previously evaluated for potential probiotic capabilities in veal calves rearing and as antagonistic agents towards multiresistant *Escherichia coli* strains. These studies are reported in appendix (chapter 8, pages: 149-153). In the third study “**Evaluation of the *in vitro* antimicrobial activity of mixtures of *Lactobacillus sakei* and *L. curvatus* isolated from Argentine meat and their application on vacuum-packed beef.**” two specific mixtures (one *L. sakei*-based mixture and one *L. curvatus*-based mixture) obtained mixing strains isolated from Argentine long shelf-life vacuum packaged beef, were evaluated *in vitro* for their antimicrobial activity against spoilage and potential pathogenic microorganisms. Moreover, the effect of the addition of the two mixtures to Italian sliced vacuum-packed beef was investigated, considering microbiological and physical-chemical parameters. Finally, in the fourth study “**Quality and hygiene of beef burgers in relation to the addition of sodium ascorbate, sodium citrate and sodium acetate**” the stabilization effects of two additive mixtures composed by sodium ascorbate, sodium citrate and different concentrations of sodium acetate were evaluated on the microbiological and physical-chemical characteristics of non-prepacked beef burgers stored in air at different temperatures.

CHAPTER 3

First study:

Effect of the Lactic Acid Bacteria on the control of listerial activity and shelf-life of smoked salmon scraps

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3. Effect of the Lactic Acid Bacteria on the control of listerial activity and shelf-life of smoked salmon scraps

AUTHORS: *Cristian Bernardi, Barbara Ripamonti, Simone Stella, Erica Tirloni, Carla Bersani, Patrizia Cattaneo.*

Dipartimento di Scienze e Tecnologie Veterinarie per la Sicurezza Alimentare. Università degli Studi di Milano.

3.1 Abstract

The potential protective activity of lactic acid bacteria (LAB) from cold smoked salmon scraps was evaluated towards *Listeria monocytogenes*. Seventy-three LAB strains were isolated, and identified by biomolecular methods; *Lactobacillus curvatus* and *Lactobacillus sakei* prevailed. Three of the strains tested, identified as *L. sakei*, profile O, had a significant inhibitory activity against *L. monocytogenes* ATCC 19115 and clones DUP-1042 and DUP-18596. The evolution of microbial populations, chemical parameters were determined at time intervals in order to verify the shelf-life. *L. monocytogenes* was isolated in half the packages also exceeding the legal limit. The shelf-life of scraps was set at 30 days. Clonal characterization of *L. monocytogenes* was performed by ribotyping. DUP-1042, one of the human pathogen clones, was the most represented pattern. The results suggest further studies aimed at the selection of autochthonous non-spoilage LAB strains as bioprotective agents for cold smoked salmon.

3.2 Introduction

Smoked salmon scraps are small bits and pieces obtained as off-cuts of the cold smoked salmon (CSS) fillet from processors' trimming and slicing procedure. Some companies often produce scraps on purpose, dicing whole smoked fillets, in order to fulfil the strong demand of this product. Scraps are increasingly used in public catering to prepare pasta sauces, pasta fillings, pizzas, lasagne, pâté, mousse, as they have similar quality characteristics of CSS at a lower price. Scraps are now widely available on retail and are very appreciated for home consumption. CSS has to be considered a perishable product and CSS slices available on the Italian market was proved to have a too long shelf-life, revealing

a very low quality of most samples at the expiry date (considering chemical and microbiological parameters) and showing spoilage signs in 50% samples at half shelf-life (Bernardi *et al.* 2009). Salmon scraps should be considered even more perishable seafood than CSS, as during the processing procedures they are submitted to more intense splitting and handling, increasing the possibility of contamination. Therefore it is important to verify the quality and the durability of smoked salmon scraps with the purpose of warranting a good qualitative and hygienic level for the whole shelf-life of the product. The shelf-life of CSS is mainly influenced by the development of specific microflora; although a clear link has not yet been established between spoilage characteristics and specific microbial species, it has been cleared that a total bacterial count around 10^7 - 10^8 CFU/g is generally observed at the sensory rejection point (Gram *et al.* 2002). Under the packaging conditions of CSS, LAB represent typically the dominant microflora, and they have a central role in the microbial events occurring in the product (Leroi, 2010). They could be present in high numbers without exerting any effect (Leroi *et al.* 2001), or, in some cases, cause evident spoilage of the product (Gonzalez-Rodriguez *et al.* 2002; Paludan-Müller *et al.* 1998), but they may even exert a bioprotective activity towards other spoilage microorganisms and several pathogenic bacteria (Brillet *et al.* 2005; Tomé *et al.* 2008a). This protective effect could be achieved by different mechanisms: the most frequently described is the acidification of the environment due to the production of organic acids, specifically lactic acid, even if other mechanisms are reported (production of hydrogen peroxide, diacetyl and bacteriocins, competitive exclusion or depletion of specific nutrients). Between the pathogenic bacteria potentially present in cold smoked fish products, *Listeria monocytogenes* has certainly the major importance. This microorganism is frequently isolated from CSS (EFSA, 2010; Meloni *et al.* 2009; Beaufort *et al.* 2007), but seafood are not frequently associated with human listeriosis (Norton *et al.* 2001). This is due to the presence of different clonal groups, which can differ in their pathogenic potential (Wiedmann *et al.* 1997). The main purpose of this study was to evaluate hygienic and qualitative aspects of CSS scraps, considering in particular the role of LAB; the characterization of LAB population was performed, in order to reveal its potential biopreservative action, with particular attention to anti-listerial activity. Besides, the durability of salmon scraps was evaluated considering the effect of microbial population on chemical parameters and volatile compounds production during the whole shelf-life of the product. Even if in a previous study (Bernardi *et al.* 2009) the volatile compounds analyses revealed itself not suitable as a single parameter for quality control of CSS, we decided to apply SPME – Gas Chromatography/Mass Spectrometry to test if it could give some useful information about scraps, on the hypothesis that they were more

perishable than CSS, as for a possible early and larger occurrence of some spoilage compounds.

3.3 Materials and methods

3.3.1 Scraps samples

Gutted Atlantic salmon, farmed in Norway, was received chilled by the processor, a medium industry of Northern Italy. On the second day from arrival, the salmon was filleted, trimmed, dry salted and left in salt for 16 hours. After that, the salmon fillets were laid on trays and left to drain at about 2°C for about 5 hours and then cold smoked. The finished product was diced and the salmon scraps obtained were immediately vacuum packaged in 500 g retail packs (rigid container in plastic bag) with a use by date of 60 days at T < 4°C.

3.3.2 Experimental design

Salmon scraps packages were directly supplied by the producer, stored at 3°C and analysed at time intervals until and beyond their expiry date: salmon scraps were sampled on days 3, 16, 30, 44, 52, 60, 66 from packaging, being 0 the day of packaging. At the established dates, the analyses were separately executed on four packs chosen at random. After the sampling for the microbiological analyses, TVB-N and physical-chemical parameters were determined. The GC/MS analyses were performed at times 16, 30, 44, 60, 66 days. At half shelf-life (HSL, 30 days), Lactobacilli strains from each pack were isolated, identified and evaluated for inhibitory activity towards *L. monocytogenes* strains previously isolated from the same product.

3.3.3 Microbiological analyses

For microbial counts, 10 g of each sample were homogenized in 90 ml of diluent solution (0.85% NaCl and 0.1% peptone), and then serial 10-fold dilutions were made in sterile saline. Total psychrotrophic count (TPC) was determined using a spread plate technique on Plate Count Agar (Oxoid, Basingstoke, UK); plates were incubated at 15°C for 5 days. Total mesophilic count (TMC) was determined according to NF EN ISO 4833:2003 method. The results obtained by TMC and TPC were compared in order to evaluate the suitability of TMC, which represents a significantly faster method, as a standard for CSS analysis. Correlation coefficients and linear regression trend lines were calculated and plotted using Microsoft Excel 2003 (Microsoft Corp., Redmond, WA). The number of *Enterobacteriaceae* was determined according to the NF EN ISO 21528-2:2004 method; *E. coli* counts were determined according to the NF EN

ISO 16649-2:2001 method, and coagulase positive Staphylococci were determined following the AFNOR 3M 01/9-04/03 method. Psychrotrophic *Pseudomonas* spp. counts were performed on Pseudomonas Agar Base added with Pseudomonas CFC Supplement (Oxoid), incubated at 7°C for 5 days. Lactobacilli were enumerated on de Man-Rogosa-Sharpe agar (Oxoid), at pH 5.5. Plates were incubated at 20°C for 3 days under anaerobic conditions (AnaeroGen, Oxoid). At half shelf-life, 20 LAB colonies for each pack were picked randomly from MRS plates containing 10-100 colonies. Presumptive Lactobacilli were sub-cultured (20°C +/- 1°C, 48 hours in anaerobiosis) in APT broth (Difco, Detroit, USA) and plated again onto MRS agar (20°C +/- 1°C for 48 h in anaerobiosis), in order to obtain pure strains. Provisional identification of genera was made on the basis of Gram stain reaction, cytochrome oxidase and catalase reactions. Gram-positive, oxidase-negative and catalase-negative microorganisms were chosen for the identification. *Salmonella* spp. detection was performed by the method NF EN ISO 6579:2002. Detection and enumeration of *L. monocytogenes* were performed according to AFNOR methods (AFNOR BRD 07/4-09/98 and AFNOR BRD 07/05-09/01). Typical colonies on Rapid'L.mono (BIO-RAD, Richmond, USA) were subcultured (37°C +/- 1°C, 48 h) on Tryptic Soy Broth (TSB) (Oxoid) and plated onto Brain Heart Infusion (BHI) agar (Oxoid). The plates were incubated at 37°C +/- 1°C for 16 +/- 2 h.

3.3.4 Identification of LAB and *L. monocytogenes* strains

The selected strains of LAB (73) were submitted to Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) with primers M13 and D11344, as previously reported by Andrighetto *et al.* (2002). Grouping of the RAPD-PCR profiles was obtained with the Gel Compar 4.1 software package (Applied Maths, Kortrijk, Belgium), using the Pearson product moment correlation coefficient and UPGMA cluster analysis. For comparison purposes, *L. curvatus* LMG 9198 and *L. sakei* ATCC 15521 type strains were used. The isolates that could not be identified by this method were submitted to DNA sequencing of V3-V8 region of 16S rDNA. For this purpose, DNA was amplified using primer pair P1 (Muyzer *et al.*, 1993) and L1401 (Zoetendal *et al.*, 1998); after purification, PCR products were sequenced. The species attribution was performed after BlastN alignment (<http://www.ncbi.nlm.nih.gov/BLAST>) of the obtained sequences with the public database available from the National Centre for Biotechnology Information (NCBI). A total of 17 isolates of *L. monocytogenes* obtained from scraps samples were submitted to ribotyping, as described by Bruce (1996). Each pattern obtained was analysed by BioNumerics version 2.5 software (Applied maths, Saint-Martens-Latem, Belgium) and

compared by the unweighted pair group method, using arithmetic average (UPGMA) algorithm.

3.3.5 *Anti-listerial activity*

The inhibitory action of the 73 LAB strains was investigated against two *L. monocytogenes* strains isolated from the samples and *L. monocytogenes* ATCC 19115, using the method by Rebucci *et al.* (2007), with minor modification. *L. monocytogenes* strains were grown aerobically overnight at 37°C in 10 ml of TSB; after incubation, the inoculated broths were added with glycerol (15% v/v) and frozen at -25°C. For each strain, an aliquot of the thawed broth was plated onto Tryptic Soy Agar (TSA) for the evaluation of the bacterial concentration. LAB were grown anaerobically at 37°C for 48 h in 10 ml of MRS broth. In order to obtain a stationary phase culture, 100 µl of broth culture were subcultured in MRS broth. LAB suspensions were seeded by drawing a cross onto the surfaces of MRS agar plates using sterile swabs; the plates were incubated for 48 h at 30°C in an anaerobic jar (Oxoid). 0.2 ml of *L. monocytogenes* suspensions (approximately 10⁷ cells) were added to 5 ml of semisolid agar (BHI broth + agar 7%) maintained in a water bath and then poured over the LAB plates. After aerobic incubation at 37°C for 24 h, the plates were checked for inhibition zones. If the LAB strain inhibited *L. monocytogenes*, a clear zone of inhibition was observed around the LAB colonies.

Subsequently, the potential production of antimicrobial compounds by LAB strains was evaluated: the three *L. monocytogenes* strains previously tested were grown in TSB; sterilized TSA was poured into Petri dishes and allowed to set. One ml of an exponential culture of *L. monocytogenes* was mixed with 7 ml of soft agar (0.7%) and poured immediately over the surface of the TSA plates. LAB isolates which had previously showed an inhibitory activity on *L. monocytogenes* were subcultured in MRS broth and incubated overnight at 37°C. The cultures were then centrifuged at 10000 rpm for 10 min at 4°C; the pH of the supernatant was measured (digital pHmeter Hamel 334B) and the absence of cells was confirmed by count on MRS agar. Wells were made on the agar surface using sterile borer (diameter 3 mm) and 50 µl of the supernatant were added to each well. The plates were incubated at 37°C for 24–48 h and evaluated for the presence of an inhibition zone; each test was performed in triplicate.

3.3.6 *Physical-chemical analyses*

Total volatile basic nitrogen (TVBN) (Reg. (EC) N. 2074/2005) was determined on each pack after sampling for microbiological analyses. Water (A.O.A.C., 1990) and salt content (Pearson, 1973) were measured on a total of 28 packs.

Water Phase Salt content (WPS) was then calculated with the formula $WPS = \frac{\% \text{salt}}{\% \text{salt} + \% \text{moisture}} \times 100$ (Huss *et al.* 1995).

3.3.7 Solid – Phase Microextraction (SPME) Headspace Analysis

A total of 20 samples were submitted to GC-MS. Sample preparation. Headspace vials were baked at 250°C for 6 hours, and then cooled at room temperature before use. Frozen smoked salmon was homogenized and 5 g per vial were put in headspace vials (20 ml) which were sealed with polytetrafluoroethylene (PTFE)-coated silicone rubber septa (20 mm diameter). The headspace was sampled with SPME fibre 75 µm carboxen-polydimethylsiloxane (CAR-PDMS) (Superchrom-Italia) for 180 min at room temperature (Aro *et al.* 2003; Triqui and Reineccius 1995).

3.3.8 Gas Chromatography – Mass spectrometry (GC/MS) Analysis

The GC/MS analysis was performed as described in Bernardi *et al.* (2009). Volatile compounds were tentatively identified by matching mass spectral data with the Wiley and NIST reference libraries of standard compounds. The identification was confirmed by comparison of the retention times and mass spectra (MS) with available authentic standards (AS). Semi-quantification of the compounds was based on arbitrary units of peak area counts divided by 10^5 .

3.3.9 Statistical analysis

Principal component analysis (PCA) was performed on correlation matrix of the areas of the chromatographic peaks, the microbial, chemical and physical parameters measured, in order to visualize data trends and to detect possible clusters within samples, thus providing a first evaluation of the discriminating efficiency of the considered variables. Statistical analyses were performed by SAS/STAT package version 8.0 (SAS Inst. Inc., NC USA). Correlation coefficient (r) of data obtained by TMC and TPC was calculated, in order to compare analytical parameters in this product (SAS/STAT package version 8.0).

3.4 Results

3.4.1 Identification of LAB strains from cold smoked salmon scraps

A total of 73 isolates at HSL were identified by RAPD-PCR or 16S rDNA sequencing, and were classified into 16 clusters (considering a cut off limit of 0.85), as shown in figure n.1. The strains belonged to the species *L. curvatus* (32 isolates, grouped in 5 clusters), *L. sakei* (31 isolates, 8 clusters), *L. fuchuensis* (2 isolates, 1 cluster) and *Carnobacterium divergens* (7 isolates, 1 cluster). Our data agree with other studies that identified LAB microflora from CSS (Hansen *et al.* 1998). The role of these microorganisms may be ambivalent; Mejlholm and Dalgaard (2007) have already identified LAB population as one of the crucial factors for the determination of quality and safety of this typology of products. It is well documented the potential spoilage activity of some LAB, but also a potential bioprotective function could be exploited. Recent literature on CSS considers the adjunct of LAB to the product a promising approach, for a protection towards spoilage and pathogenic bacteria, with particular interest for *L. monocytogenes*. (Leroi *et al.* 1996; Katla *et al.* 2001; Brillet *et al.* 2005; Jouffraud *et al.* 2006; Tomé *et al.* 2008b).

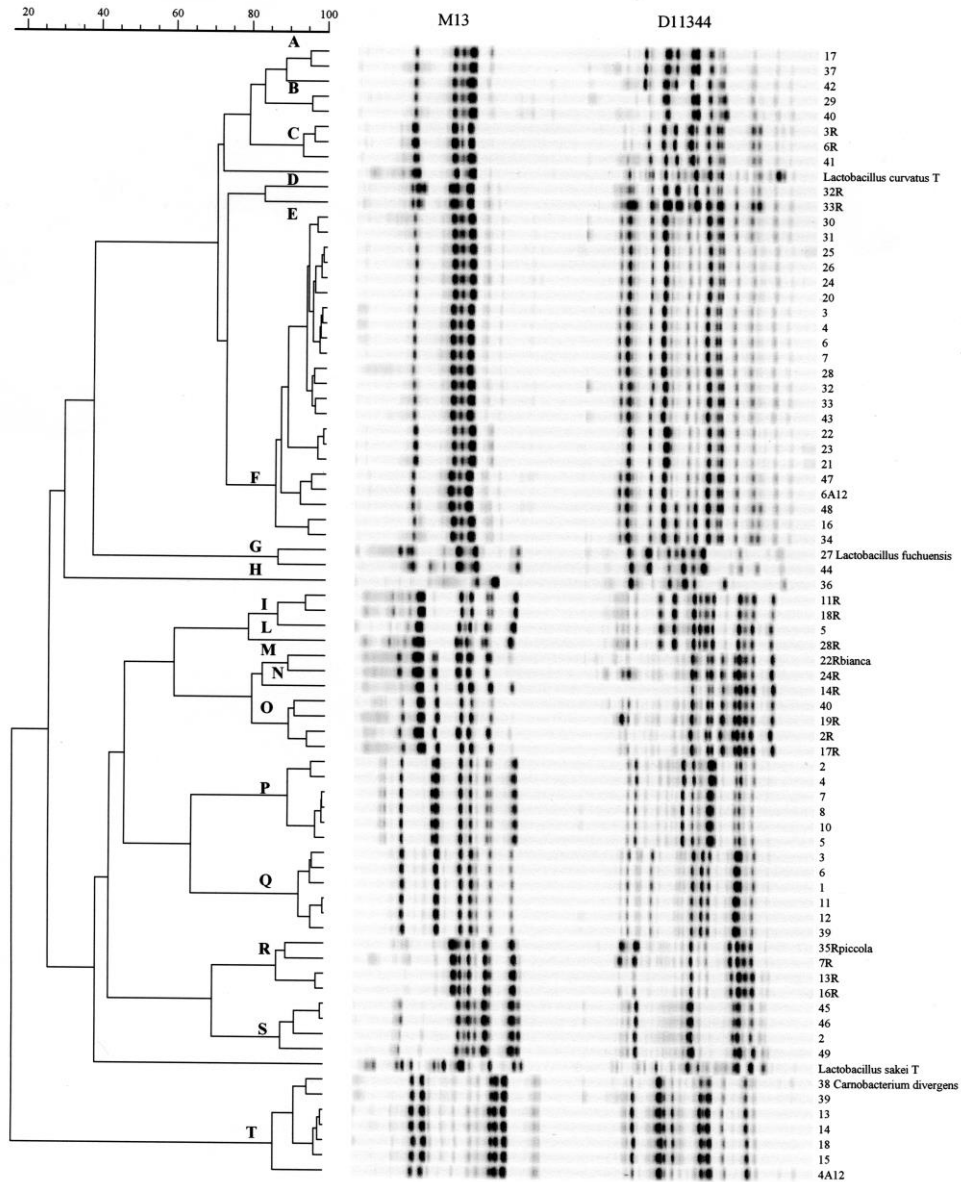


Figure 1: Cluster analysis of the 71 LAB isolates from smoked salmon scraps. Different letters indicate the different clusters: A-F *Lactobacillus curvatus*; G *Lactobacillus fuchbuenensis*, H *Lactobacillus* not identified, I-S *Lactobacillus sakei* T *Carnobacterium divergens*, Internal standard *Lactobacillus curvatus* T and *Lactobacillus sakei* T.

3.4.2 Anti-listerial activity

The anti-listeria activity of 73 LAB isolates at HSL was evaluated; *L. monocytogenes* ATCC 19115 and 2 *L. monocytogenes* clones isolated from salmon scraps, identified as DUP-1042 and DUP-18596, were used as targets. Of the LAB strains tested, only 3 (n. 3, 6 and 11), identified as *L. sakei*, profile O, showed an evident

inhibitory activity (an inhibition halo larger than 20 mm), towards the three *L. monocytogenes* strains, suggesting that anti-listerial action must be considered as a clonal characteristic. LAB have been recently evaluated by different authors as potential bioprotective agents to control the growth of *L. monocytogenes* in CSS: the most studied LAB are *Carnobacterium* spp. and *L. curvatus*, but also *L. sakei* has been recognized as a promising anti-listerial species (Brillet *et al.* 2005; Ghalfi *et al.* 2006; Weiss and Hammes, 2006; Tomé *et al.* 2008a, b). The supernatant of Lactobacilli cultures didn't show an inhibition on listerial growth; it is difficult to explain this result, as the tests conducted with viable lactobacilli showed a large inhibition area, suggesting the production of antimicrobial compounds. In particular, the acidification of the supernatant (pH 4.23 ± 0.02) should be further investigated. The lack of efficacy of the supernatant of bacterial cultures suggests that the adjunct of live lactobacilli on the product should be performed. It has to be noted that several studies have already showed the possible use of live LAB on CSS (Leroi, 2010); our results suggest the possibility of testing the activity of *L. sakei* profile O on CSS, in order to evaluate all the aspects linked to this strain, such as potential spoilage activity and anti-listerial action. *L. sakei* is known as a causative agent of sulphurous or acidic odours (Stohr *et al.* 2001), associated with the production of H₂S, acetic acid and ethyl and n-propyl acetate (Joffraud *et al.* 2001) but the spoiling capacity, such as antimicrobial activity, depends also on the strains considered, and not all *L. sakei* strains affect the sensory quality of CSS (Weiss and Hammes, 2006). Therefore, a selection of non-spoilage strains should be made before their use. Further challenge tests on the product are needed to evaluate potential spoilage activity of *L. sakei* isolates in real commercial conditions, such as their inhibitory action towards *L. monocytogenes* and spoilage microorganisms, with positive effects on the shelf-life.

3.4.3 Microbiological analyses and identification of *L. monocytogenes* isolates

Microbiological methods are commonly applied for quality and safety monitoring of CSS and microbiological parameters like TMC and TPC are used to estimate the shelf-life of the product. In particular, psychrotrophic aerobic bacteria are an important hygiene indicator, as they include potential spoilage microorganisms. The threshold value used to discriminate similar food products is 10⁶ CFU/g, that is the limit for the end of shelf-life often used in the industry (Olafsdottir *et al.* 2005). The total bacterial count at the sensory rejection point is generally around 10⁷ - 10⁸ CFU/g, although the microorganisms isolated differ considerably from one study to another, depending on the process involved in different plants and on the analytical methods applied (Hansen *et al.* 1998; Espe *et al.* 2004). At the 30th day TMC and TPC were over the threshold of 10⁶ CFU/g, considered a high contamination level even if this limit is not in

agreement with the results of sensory panellists who estimated that samples with counts of 3×10^6 CFU/g had not exceeded the limit of shelf-life (Leroi *et al.* 1998). During the storage period, TMC and TPC increased significantly reaching a plateau of about 10^8 CFU/g at 52 days (figure n.2). The correlation between TPC and TMC values was highly significant ($r = 0.93$, $p < 0.01$), confirming the results obtained previously (Cempírková, 2002) and represent an important finding in order to choose parameters in commercial check, because of their different analytical time (TMC = 48 h, TPC = 5 days). LAB population increased during the storage of the scraps, so becoming, from the 30th day, the dominant microflora; their predominance in cold smoked fish products at the end of shelf-life is widely recognised (Joffraud *et al.* 2001; Sthor *et al.* 2001; Gonzalez-Rodriguez *et al.* 2002). Lactobacilli, as other bacterial groups, have been identified as weak or strong spoilage organisms, depending on the strain (Leroi *et al.* 1999), as some of them produce sulphurous and acidic off-odours. Different Gram-negative bacteria are often present in CSS (Stohr *et al.* 2001); in our study, the numbers of *Enterobacteriaceae* were high (< 2 -3.9 Log CFU/g), even if they did not reach critical levels. The number of *Pseudomonas* spp. was constant considering the different samples and along the whole trial, with a range of 3.1-3.9 Log CFU/g. No *E. coli* or *S. aureus* significant counts were revealed (< 2 Log CFU/g); *Salmonella* spp. was not found in any of the samples.

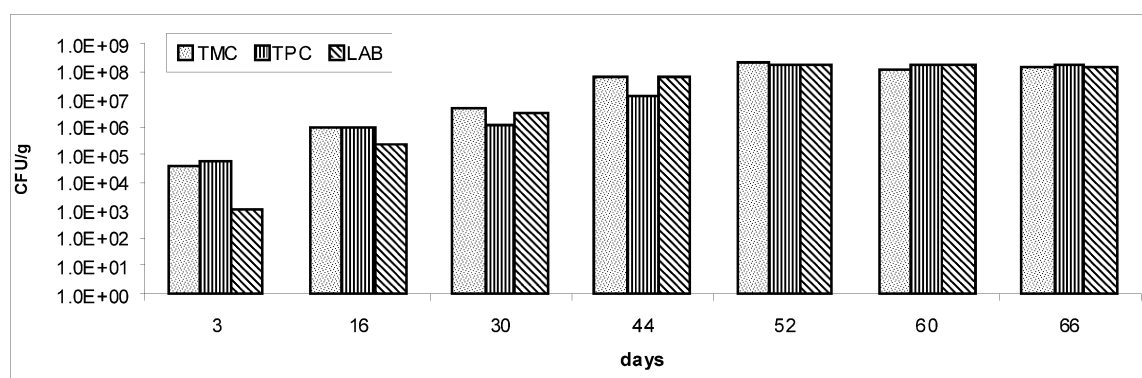


Figure 2: Total Mesophilic Count (TMC), Total Psychrotrophic Count (TPC) and Lactobacilli counts (LAB) of smoked salmon scraps during the storage period. At 30th day the TMC and TPC were over the threshold of 10^6 CFU/g.

L. monocytogenes was isolated from 14 packages of the 28 sampled in the whole trial period; specific bacterial counts of positive samples ranged from < 5 to 750 CFU/g, with only 2 samples exceeding 100 CFU/g; we couldn't observe a trend in listerial contamination levels, as the highest counts were obtained from samples at 30 and 52 days, while positive and negative samples seemed to be

casually distributed in all sampling times. These results underline the importance of the initial contamination level of this product. The analysis of *L. monocytogenes* isolates lead to the identification of three DUP-ID patterns (figure n.3), belonging to Lineages I and II, which are shown to differ in pathogenic potential (Wiedmann *et al.* 1997). DUP-1042, classified in Lineage I, was the most represented pattern (11 of 17 isolates); it is widespread distributed, being frequently isolated from human and food samples, especially seafood (Norton *et al.* 2001; Gendel and Ulaszek, 2000; Lappi *et al.* 2004; Meloni *et al.* 2009) and is one of the most important clones causing epidemic and sporadic listeriosis outbreaks (Saunders *et al.* 2006; Jeffers *et al.* 2001). The two other patterns identified were DUP-18596 (4 of 17) and DUP-1062 (2 of 17), belonging to Lineage II, which consists of low pathogenic clones considered to be associated to environment (Ramaswamy *et al.* 2007). In particular, these ribotypes seem to be linked to cold smoked fish industries (Gendel and Ulaszek, 2000; Meloni *et al.* 2009). All the isolated *L. monocytogenes* strains are shown to persist in the environment of smoked fish processing plants (Norton *et al.* 2001; Saunders *et al.* 2006; Lappi *et al.* 2004). *L. monocytogenes* confirmed its importance for the CSS industry; the most interesting but alarming finding is the high prevalence of pathogenic clones, linked to human listeriosis. It is therefore of utmost importance, in the presence of *L. monocytogenes* in the premises, verifying the clone by means of biomolecular techniques.

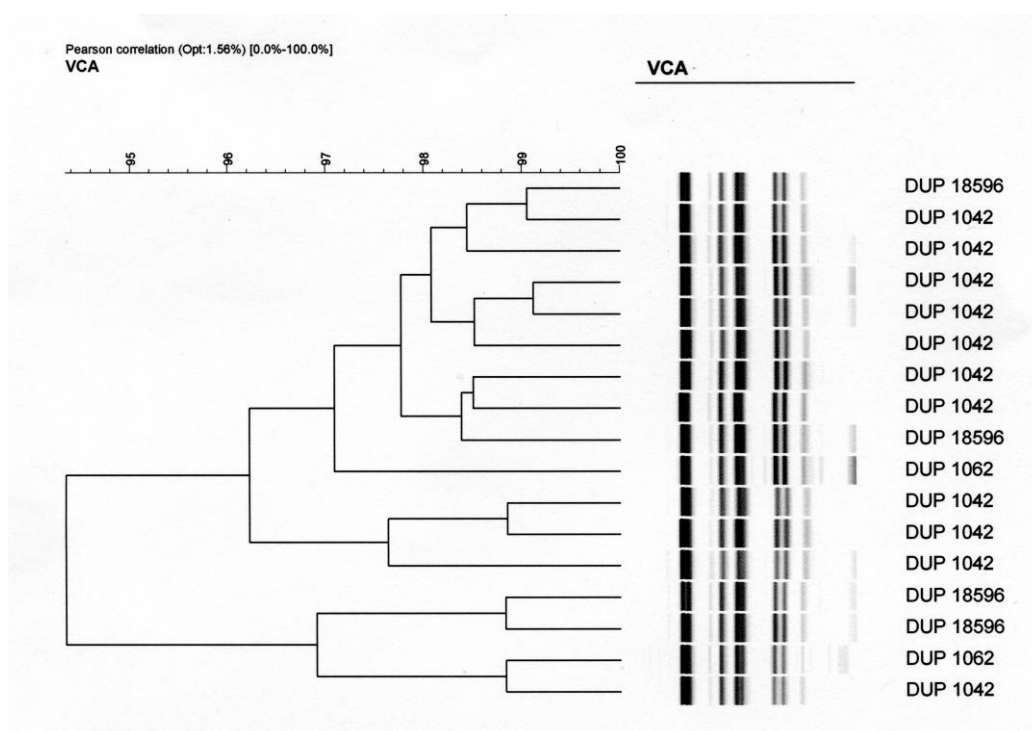


Figure 3: Dendrogram of *L. monocytogenes* isolated from smoked salmon scraps: DUP 1042 is classified in Lineage I, DUP 18596 and DUP 1062 are classified in Lineage II.

3.4.4 Physicochemical characteristics

The mean content of total chlorides (n = 27 samples) was 3.25% with a SD of 0.51; the mean WPS value was 5.11% with a SD of 0.69. The data obtained were compared with those of Espe *et al.* (2004) who reported a mean value of salt of 2.62 g/100g and mean water content of 62.5%, equal to 4.02 WPS%, on 48 French commercial smoked salmon products. Cornu *et al.* (2006) reported the means (SD) of 40 French commercial products: salt content 2.85 (0.65) g/100g, water content 61.3 (3.57) % and WPS 4.62 (0.96) %. Mean WPS of smoked salmon scraps (5.11%) was higher than the two French studies, but it was very similar to Italian commercial smoked salmon products analysed by Bernardi *et al.* (2009) who reported a mean salt content of 3.43 (0.56) % and a mean WPS value of 4.9 (0.8) %. The Center for Food Safety and Applied Nutrition, US FDA (2009) recommends a WPS limit of 3.5%, that allows control of psychrotrophic *Clostridium botulinum* in combination with a storage chill temperature (< 4.4°C). In the present study, all the samples had higher WPS value than the considered limit (minimum value observed 4.1%). The TVBN limit level of 40 mg N /100 g (as proposed by Cantoni *et al.* 1993) was reached in scraps on the 40th day from packaging. The mean TVBN values at 30° day was 36.6 mgN/100 g with a (SD = 4.68). In a previous work (Bernardi *et al.* 2009) the mean TVBN values at 30° day from packaging of smoked salmon fillets was 38.2 mg N/100 g, the difference is not significant (P=0.83).

3.4.5 Volatile compounds

The aim of volatile compounds analysis was to choose the more appropriate quality and spoilage indicators for smoked salmon scraps. The analysis of the smoked salmon samples using SPME method allowed for the identification of 31 compounds (table n.1). PCA was carried out to establish a correlation among the chemical, physical and microbiological parameters and the volatile compounds. For this purpose, all available data were included in the statistical analysis. The first two principal components explained 70.1% of the data's variance; they divided the samples in two groups: one composed by the samples with a shelf-life until 26 days and another one by the remnants. The second group was marked out by spoilage related compounds, such as: 2-butanone, 2,3 butanedione and 1-propanol. The positive correlation among 2,3 butanedione, 1-propanol and the microbial count of LAB confirms the microbial origin of these volatile compounds. In particular, Joffraud *et al.* (2001) associated the 2,3 butanedione production to the *Carnobacterium piscicola* activity, a LAB species most frequent isolated from smoked salmon. Jørgensen *et al.* (2001) demonstrated that 1-propanol and 2 butanone were microbial products, since these volatile compounds are not detected in sterile sample. Hexanal, a

secondary products of lipid oxidation, decreased till the 30th day, then it was no more found; this is in agreement with the study of Jørgensen *et al.* (2001) where hexanal decreased with spoilage. 3-hydroxy-2-butanone (acetoin) was detected in all samples, but it did not increase during storage; this molecule is considered a marker of spoilage in both fresh and smoked salmon and it is not clear why this molecule did not increase with time.

Peak number	Compound	Identification	Structure
1	carbon disulphide	MS	sulfur compound
2	acetone	MS, AS, RI	ketone
3	2-butanone	MS, AS, RI	ketone
4	ethanol	MS, AS, RI	alcohol
5	2-butanolo	MS, AS, RI	alcohol
6	2,3-pentanedione	MS, AS, RI	ketone
7	hexanal	MS, AS, RI	aldehyde
8	1 penten3ol	MS, AS, RI	alcohol
9	2 butanone 3hydroxy	MS, AS, RI	ketone
10	2 propanone 1 hydroxy	MS	ketone
11	2-cyclopenten-1-one, 2-methyl-	MS	ketone
12	1-hydroxy-2-butanone	MS	ketone
13	acetic acid	MS, AS, RI	acid
14	2-Furancarboxaldehyde	MS, AS, RI	aldehyde
15	2 cyclopenten 1 one 3 methyl	MS	ketone
16	ethanone, 1-(2-furanyl)	MS, AS, RI	ketone
17	benzaldehyde	MS, AS, RI	aldehyde
18	propionic acid	MS, AS, RI	acid
19	2-furancarboxaldehyde,5-methyl	MS	aldehyde
20	gamma-butyrolactone	MS, AS, RI	lactone
21	butanoic acid	MS, AS, RI	acid
22	furfural	MS	aldehyde
23	alkane	MS	alkane
24	phenol, 2-methoxy	MS	phenol
25	phenol	MS, AS, RI	phenol
26	Propanal	MS	aldehyde
27	alkane	MS	alkane
28	2,3 butanedione	MS, AS, RI	ketone
29	alkane	MS	alkane
30	ethylguaiacol	MS, AS, RI	phenol
31	m-cresol	MS, AS, RI	aromatic

Table 1: Volatile compounds identified in smoked salmon scraps by GC-MS. Volatile compounds were identified by comparison with reference substances based on the following criteria: retention index (RI) and mass spectrum obtained from authentic standard (AS); mass spectrum from NIST and Wiley libraries (MS).

3.4.6 Shelf-life

A Multiple Compound Quality Index (MCQI) proposed by Leroi *et al.* (2001) was applied to samples on all sampling steps. The index, developed by multiple linear regression, relates the remaining shelf-life in week (RSL) of cold smoked salmon at 5°C with the level of *Lactobacillus* and the content of TVBN, according to the model: $RSL = 4.78 - 0.34 * \text{Log} (Lactobacillus/g) - 0.06 * \text{TVBN (mg N/100g)}$. The calculated RSL were set against the days from packaging. The resulting regression line was $y = -0.0583x + 2.135$, where $y = \text{RSL}$, from which the end of shelf-life (RSL=0) is 36 days ($r = -0.09439$, $p < 0.001$, N of pairs of data 26). The established shelf-life of 60 days did not match with the analytical results. We think that, in the hygienic conditions determined by the analyses, an expiry date of no more than 30 days can be attributed to this product, considering that the test was performed at controlled temperature, the presence of *L. monocytogenes* and the specific characteristics of the scraps. At the beginning of the study, we hypothesised that salmon scraps were more perishable than sliced CSS because more cuts increase the possibility of a higher microbial growth, due to a higher contamination, a wider exposed surface, a higher presence of meat juices, the cut itself that generates heating. Even if the mean TPC at 30 days in scraps was higher than what determined previously in CSS (geometric mean $8.4 * 10^4$ CFU/g) (Bernardi *et al.* 2009), the calculated RSL did not show differences between this production and sliced CSS. This finding could be attributed to the fact that the samples were obtained dicing whole fillets and not collecting pieces from trimming and slicing, procedure with an even higher possibility of microbial growth. However, it has to be considered that CSS, due to its physicochemical characteristics, supports the growth of *L. monocytogenes*, and published data show a significant prevalence (10-20%) of the pathogen in smoked seafood (Gnanou Besse *et al.* 2004; Beaufort *et al.* 2007; EFSA, 2010). So, the definition of a sufficiently short shelf-life should be considered as a precautionary factor in order to avoid the raise of bacterial numbers which could make the product potentially dangerous for the consumer and lead to its withdrawal, in accordance with European Union legislation.

3.5 Conclusions

In this study some important factors which influence safety, quality and durability of smoked salmon scraps are focused. A highly pathogenic *L. monocytogenes* clone was identified in CSS samples; this finding underlines the importance of interventions aimed to reduce the risk of food contamination in CSS industry and to prevent the replication of *L. monocytogenes* using microorganisms with a potential biopreservative action. Anti-listerial activity of *L. sakei* strains was observed, even if the nature of this activity should be further

investigated. This study shows that a shelf-life of 60 days was too long, as an expiry date of no more than 30 days should be attributed to this kind of product. Volatile compounds analysis did not allow to determine shelf-life and did not seem a suitable method for a rapid and simple quality control.

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CHAPTER 4

Second study:

***In vitro* evaluation of *Lactobacillus animalis* SB310, *Lactobacillus paracasei* subsp. *paracasei* SB137 and their mixtures as potential bioprotective agents for raw meat**

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4. *In vitro* evaluation of *Lactobacillus animalis* SB310, *Lactobacillus paracasei* subsp. *paracasei* SB137 and their mixtures as potential bioprotective agents for raw meat

AUTHORS: *Erica Tirloni, Patrizia Cattaneo, Barbara Ripamonti, Alessandro Agazzi, Carla Bersani, Simone Stella*

Department of Health, Animal Science and Food Safety, Università degli Studi di Milano, via Celoria 10, 20133, Milan, Italy

4.1 Abstract

Lactobacillus animalis SB310 and *Lactobacillus paracasei* subsp. *paracasei* SB137, isolated from the gut of veal calves, were investigated in terms of antimicrobial activity for a possible application as bioprotective agents in vacuum-packed raw meat. In the first trial, cultures of single strains and their mixture obtained adding the two strains before the incubation, their cell-free supernatants and buffered cell-free supernatants were tested *in vitro* against a wide range of spoilage or potentially pathogenic bacteria. In the second trial different mixtures were evaluated for the same tests (*L. animalis*: *L. paracasei* rates = 1:2, 1:5, 1:10, 2:1, 5:1, 10:1 and 1:1 obtained mixing the two cultures grown separately). An evident inhibitory activity exerted by the single *Lactobacillus* strains and the mixtures was observed for all the target bacteria tested. In almost all the cases, the inhibition halos produced by the mixtures were significantly higher than those produced by the single strains, especially if *L. paracasei* subsp. *paracasei* SB317 was predominant in the mixture. Among the target bacteria, *Pseudomonas fluorescens* and *P. putida* were clearly the most susceptible; a high variability in *Enterobacteriaceae* was detected, depending on the species. *Serratia marcescens* and *E. coli* O157:H7 resulted as the less susceptible strains. A very limited activity of the cell-free supernatants was found for all the 12 strains tested, compared with the action of viable Lactobacilli, highlighting that the antimicrobial action originated from a combination of bacterial competition and the production of extracellular compounds. The absence of effects by the buffered cell-free supernatants suggests that these compounds are organic acids. Further studies are necessary to clarify the effects of these strains when applied to raw meat.

4.2 Introduction

A growing number of consumers demand safe, durable but minimally processed food, prepared with the least amount of chemical preservatives and additives. Techniques applied to raw meat need to preserve its sensorial shelf life (particularly the flavour and colour) and delay the critical growth levels of pathogenic microorganisms (“safe shelf life” as defined by Dalgaard, 2009), without modifying the characteristics of the fresh product (Stiles, 1996; Lücke, 2000; Castellano & Vignolo, 2006; Dortu, Huch, Holzapfel, Franz, & Thonart, 2008; Rodgers, 2008).

Several researchers have focused on the effects of “sensory milder thermal technologies”, especially on the application of bioprotective cultures, with promising effects (Aymerich, Picouet & Monfort, 2008; Dortu, Huch, Holzapfel, Franz, & Thonart, 2008; Castellano & Vignolo, 2006; Budde, Hornbaek, Jacobsen, Barkholt, & Koch, 2002). Biopreservation consists in inoculating food products with specific microbial strains that inhibit, directly or by producing metabolites, the growth of undesirable spoilage and potential pathogenic bacteria (Lücke, 2000). Lactic acid bacteria (LAB) have a major potential as biopreservants, with a long history of harmless use in foods as they dominate the natural microflora of meat products, vegetables, milk and fish products during storage and are considered to be GRAS (Generally Recognized As Safe) (Aguirre & Collins, 1993). The antagonistic ability of LAB can be explained by the competition for nutrients and through the production of antimicrobial compounds such as bacteriocins, reuterin, organic acids (mainly acetic and lactic acids), carbon dioxide, diacetyl, ethanol, hydrogen peroxide and enzymes. To be applied for the biopreservation of raw meat, LAB cultures must survive at refrigeration temperatures (0-4°C), compete with the relative high indigenous microbial load of raw meat, actively inhibit pathogenic and specific spoilage bacteria, and do not alter the sensory properties of the meat. Some studies have also revealed the potentially positive activity exercised by LAB enzymes in improving meat flavour, tenderness and nutritional quality during vacuum storage (Fadda et al., 1999; Castellano, Gonzáles, Carduza & Vignolo, 2010).

In a previous work, lactic acid bacteria, isolated from the gut of veal calves, were evaluated for probiotic capabilities, by selecting three microorganisms in order to develop a species-specific multistrain mixture (*Lactobacillus animalis* SB310-*Lactobacillus paracasei* subsp. *paracasei* SB137-*Bacillus coagulans* SB117). *L. animalis* SB310 and *L. paracasei* subsp. *paracasei* SB137, showed promising probiotic capabilities, an absence of mutual antagonistic activity, and an acidification capability due to the production of organic acids (acetic and lactic acid) (Ripamonti et al., 2011). The same mixture was then successfully tested *in vitro* against multiresistant *E. coli* isolates from veal calves feces (Ripamonti et al., 2013).

In this study we investigated two of these microorganisms (*L. animalis* SB310 and *L. paracasei* subsp. *paracasei* SB137), which are able to survive in anaerobic conditions. Our aim was to test their antimicrobial activity in relation to a wide range of spoilage or potentially pathogenic bacteria, for a possible use as bioprotective agents in vacuum-packed raw meat.

4.3 Materials and methods

4.3.1 Preparation of *Lactobacillus* strains

Lactobacillus animalis SB310 and *L. paracasei* subsp. *paracasei* SB137 were stored in cryovials (Microbank™, Pro-Lab Diagnostics, Richmond Hill, Canada) at -70°C until their use. The strains were subcultured in MRS broth tubes (de Man–Rogosa–Sharpe broth, Oxoid, Basingstoke, UK) and incubated at 30°C for 48h in jars (Anaerojar, Oxoid) with anaerobiosis generators (AnaeroGen, Oxoid).

4.3.2 Antimicrobial activity against spoilage and potential pathogenic microorganisms - First trial

The first trial was conducted in order to evaluate the antimicrobial activity of the individual strains and of their 1:1 mixture. A volume of 100 µl of each broth culture were singularly inoculated in 10 mL MRS broth tubes and incubated at 30°C for 48h in anaerobiosis. The optical density (OD) was recorded at the moment of the incubation and the precultures were collected after 48h in exponential growth phase, defined as a relative change in absorbance of at least 0.2 at 540 (approximately the strains were collected when OD was close to 0.5/0.7 at the end of the 48h); if needed, the precultures were diluted prior to perform the tests. In addition, in order to test the combined activity of the two strains, 50 µl of each suspension (OD adjusted) were inoculated in MRS broth tubes, incubated at 30°C for 48h in anaerobiosis (mixture 1:1a). After incubation, each broth was OD adjusted (about 0.5) and spotted with a sterile swab (Carlo Erba, Rodano, Italy) on the surface of the MRS agar plates, which were subsequently incubated for 48 h at 30°C in an anaerobic jar. A selection of 12 spoilage or pathogenic microorganisms was used as targets for the tests: *Escherichia coli* ATCC 25922, *Escherichia coli* 0157:H7 DSM 13526, *Proteus vulgaris* ATCC 8427, *Salmonella* Typhimurium ATCC 14028, *Serratia marcescens* ATCC 14756, *Yersinia enterocolitica* ATCC 23715, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas fluorescens* ATCC 13525, *Pseudomonas putida* ATCC 49128, *Listeria monocytogenes* ATCC 7644, *Listeria innocua* ATCC 33090, *Staphylococcus aureus* ATCC 6538. Each strain was subcultured aerobically overnight at 37°C (30°C for *P. fluorescens* and *P. putida*) in 10 mL TSB tubes (Tryptic Soy Broth, Oxoid). The optical density (OD) was recorded at the moment of the incubation and the

precultures were collected after 24h in exponential growth phase, defined as a relative change in absorbance of at least 0.2 at 540 (approximately the strains were collected when OD was close to 0.5/0.7 at the end of the 48h); if needed, they were diluted prior to perform the tests. For each microorganism, 0.2 mL of bacterial suspension were added to a 5 mL share of semisolid agar (BHI, Brain Heart Infusion Broth, Oxoid + agar 0.7%), maintained in a water bath (45°C) and then poured over the MRS plates previously spotted with *Lactobacillus animalis* SB310, *L. paracasei* subsp. *paracasei* SB137, or their mixture. To avoid the dispersion of Lactobacilli from the spot in the BHI, a small amount (3-4 drops) of semisolid agar already inoculated was firstly dispensed using a sterile Pasteur pipette (Carlo Erba) onto the surface of the spot; after solidification (approximately 3 minutes at room temperature), the remaining BHI was poured onto the plates. After aerobic incubation at 37°C (30°C for *P. fluorescens* and *P. putida*) for 24 h, the plates were checked. A clear zone around the *Lactobacillus* spot indicated the inhibition of the target microorganisms. The mean rays of the inhibition halos (distances from the spotted inoculum and the growth of target microorganism) were measured and expressed in mm.

4.3.3 Antimicrobial activity of cell-free supernatants against spoilage and potential pathogenic microorganisms

In order to determine whether the inhibition was due to the production of antagonistic compounds, the cultural Lactobacilli cell-free supernatants were tested against the same 12 bacteria. The mixture and each single *Lactobacillus* strain were subcultured in MRS broth as described above. After 48 h of incubation, an aliquot of each culture was centrifuged at 7700 rpm for 10 min. The supernatants obtained were subsequently filtered using 0.2 µm filters (Sacco, Cadorago, I) and maintained at 4°C. For each broth, pH was measured by a pH meter (Ghiaroni, XS pH6, Buccinasco, I): three independent measurements were performed on each sample and means were calculated. Each of the 12 target strains was inoculated into 10 mL TSB tubes and incubated as described above; 1 mL of inoculated TSB was then transferred into 20 mL flasks of Tryptic Soy Agar (Oxoid), maintained in a water bath at 45°C, carefully mixed, and poured onto sterile Petri plates (Carlo Erba). Once the media had solidified, blank discs (Oxoid) were dipped with the supernatant of each *Lactobacillus* strain and of the mixture. They were placed onto the plates, and incubated at 37°C (30°C for *P. fluorescens* and *P. putida*) for 24 h. Clear zones around the discs were recorded. Finally, in order to evaluate whether any inhibition was due to the production of organic acids, the pH of cell-free supernatants was adjusted to 6.5 with NaOH (1 N) (Sigma, Milano, I) and the same test was performed. All the tests were repeated six times, and mean values of the inhibition halos were calculated.

4.3.4 Second trial

In order to attest the inhibitory effect of the two strains blended together, different mixtures were tested. The precultures of each *Lactobacillus* strain were prepared and eventually diluted as reported above and at the moment of the test were mixed proportionally as reported in table 1. The single strains and the different mixtures obtained were submitted to the same tests described above in section 2.2.1 and 2.2.2 for the evaluation of antimicrobial capabilities.

Mixture	Composition
A	100% <i>L. animalis</i>
10:1	91% <i>L. animalis</i> , 9% <i>L. paracasei</i>
5:1	83% <i>L. animalis</i> , 17% <i>L. paracasei</i>
2:1	67% <i>L. animalis</i> , 33% <i>L. paracasei</i>
1:1b*	50% <i>L. animalis</i> , 50% <i>L. paracasei</i>
1:2	33% <i>L. animalis</i> , 67% <i>L. paracasei</i>
1:5	17% <i>L. animalis</i> , 83% <i>L. paracasei</i>
1:10	9% <i>L. animalis</i> , 91% <i>L. paracasei</i>
P	100% <i>L. paracasei</i>

Table 1
Experimental design.

*1:1b mixture prepared adding the two strains after 48h of incubation.

4.3.5 Statistical analysis

The experimental data from inhibition halos were analyzed by a GLM procedure (SAS Inst. Inc., Cary, NC, 2006) as a randomized complete block design; the model included the *Lactobacillus* strain or the mixtures as a fixed effect both on target strains of pathogens or supernatant. Differences in inhibition halos among the target strains of pathogens within each *Lactobacillus* strain or the mixtures were also checked by a GLM. Moreover, for *L. animalis* and *L. paracasei* single cultures data, results obtained from trial 1 and 2 were analyzed by a GLM procedure. For all statistical evaluations, threshold levels of $P \leq 0.05$ and $P \leq 0.01$ were considered for significance.

4.4 Results

4.4.1 Trial 1 - Antimicrobial activity of viable *Lactobacilli* against spoilage and potential pathogenic microorganisms

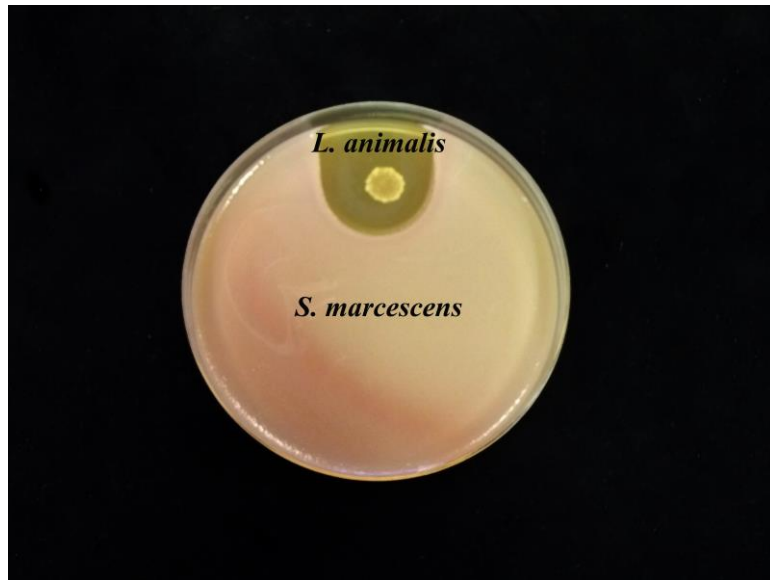
The selection of beneficial microbes as biopreservatives for food products is based mainly on their antimicrobial activity against spoilage and pathogenic microorganisms. This characteristic was revealed for both *L. animalis* SB310 and *L. paracasei* subsp. *paracasei* SB137 in the first trial. Although several *Lactobacilli* have been tested over the last few decades for their antagonistic activity against the most common food-borne pathogens, only limited knowledge regarding these two microorganisms can be found in the literature (Caridi, 2002; Jin, Marquardt & Baidoo, 2000). The mean radii of the inhibition halos are reported in Table 2. We found that the individual *Lactobacillus* strains and the mixture exerted an inhibitory activity against all the bacteria tested (93.6% of all the halos were >10 mm). In almost all the cases, the effect of the mixture was greater than those exerted by the individual *Lactobacilli*. A highly significant difference was detected between the mixture and *L. animalis* for all the target strains tested except for *E. coli* O157:H7, *L. innocua*, *P. fluorescens* and *P. putida*. In particular, highly significant differences were recorded for *S. marcescens* (P=0.0007), *Y. enterocolitica* (P=0.0005) and *S. aureus* (P=0.0009). In addition, 58.3% of the plates inoculated with the mixture showed a halo > 20 mm, while the rates detected for *L. paracasei* and *L. animalis* were only 30.6% and 16.7%, respectively. This could be related to the possible presence of a synergic action of the two *Lactobacillus* strains. Positive interrelationships between microorganisms in terms of additive effects of the specific properties of each one and/or a mutual exchange of beneficial metabolites, are well known and can lead to an improvement in their biological activity (Timmerman, Koning, Mulder, Rambouts & Beynen, 2004).

L. paracasei subsp. *paracasei* SB317 showed a higher growth rate and antimicrobial activity than *L. animalis* SB310. In fact, in the broth inoculated with *L. paracasei* subsp. *paracasei* SB317 at the end of the 48h of incubation and before the spread onto the plates an OD of 1.315 was registered, while *L. animalis* reported an OD of 0.675; the OD of the mixture registered was 1.022. *L. paracasei* subsp. *paracasei* consistently produced wider halos than *L. animalis*; in particular this difference was significant for *P. vulgaris* (P=0.0482), *S. marcescens* (P=0.0190), *Y. enterocolitica* (P=0.0062) and *L. monocytogenes* (P=0.0113). A marked difference between the target bacterial species was also observed. Generally, Gram negative bacteria were more susceptible than Gram positive bacteria, producing a mean halo of 31.27 mm vs 19 mm with the mixture. An efficient inhibitory action was detected against all the strains of *Pseudomonas* spp.; according to Moore et al. (2006), most of the species of this genus fails to grow under acid conditions. *P. aeruginosa* proved to be the most resistant *Pseudomonas* strain tested in our study while *P. fluorescens* and *P. putida* were the most susceptible species, as no growth was

detected within the plates. In this case, an arranged 65 mm value was attributed to the halos, resulting in a highly significant difference against all the other strains tested.

Enterobacteriaceae were susceptible to the Lactobacilli, with significant differences among the species. *Serratia marcescens* (Figure 1) and *Escherichia coli* O157:H7 were the most resistant, while very large inhibition halos were observed for *Y. enterocolitica* and *P. vulgaris*. The activity of lactic acid bacteria against *Enterobacteriaceae*, in particular *E. coli*, has been investigated with varying results mainly due to interspecific and intraspecific differences between the strains tested (Ammor, Tauveron, Dufour & Chevallier, 2006; Lavermicocca, Valerio, Lonigro, Di Leo & Visconti, 2008; Awaisheh & Ibrahim, 2009; Liu et al., 2013). Only two previous studies have investigated the activity of *L. paracasei* against *E. coli*. Caridi (2002) showed an intensive antagonistic activity of *L. paracasei* subsp. *paracasei* against three *E. coli* isolates from cheese. In addition, Jin, Marquardt & Baidoo (2000) found that one *L. paracasei* strain was the most effective of 14 *Lactobacillus* strains tested against enterotoxigenic *E. coli*, producing inhibition zones ranging from 6.9 to 11.4 mm, which are slightly lower than our results, which ranged from 9.0 to 18.0 mm.

Of the Gram positive bacteria, *Listeria* spp. was found to be the least susceptible. No significant differences were revealed between *L. monocytogenes* and *L. innocua*, thus confirming their high metabolic similarity. Several authors have tackled the potential inhibition of this microorganism by LAB, as *L. monocytogenes* represents one of the major potential health concerns in vacuum-packaged refrigerated meat, revealing potential beneficial effects in almost all the studies (Vaughan et al., 1994; Ammor, Tauveron, Dufour & Chevallier, 2006; Jones, Hussein, Zagorec, Brightwell & Tagg, 2008; Awaisheh & Ibrahim, 2009). *S. aureus* showed a high susceptibility to the mixture. Contrasting data have been reported concerning its sensitivity to lactic acid bacteria, thus confirming its moderate ability as a competitor (Ammor, Tauveron, Dufour & Chevallier, 2006; Vaughan et al., 1994).



a)



b)

Figure 1: Inhibition test of *Serratia marcescens* (a) by *L. animalis* SB310 and *Pseudomonas aeruginosa* (b) by the mixture *L. animalis* SB310–*L. paracasei* subsp. *paracasei* SB137 (1:1).

Target strains	<i>L. animalis</i> SB310	<i>L. paracasei</i> subsp. <i>paracasei</i> SB137	Mixture 1:1a*
<i>Escherichia coli</i>	13.3±0.6 ^b	14.0±2.6 ^b	18.0±0.0 ^a
<i>Escherichia coli</i> O157:H7	9.0±5.6	15.5±2.1	16.7±3.8
<i>Proteus vulgaris</i>	14.7±1.5 ^b	21.3±4.2 ^a	23.0±3.6 ^a
<i>Salmonella</i> Typhimurium	14.7±2.5 ^b	16.0±1.7	20.3±3.1 ^a
<i>Serratia marcescens</i>	9.7±2.1 ^{B,c}	14.3±1.5 ^b	19.0±1.7 ^{A,a}
<i>Yersinia enterocolitica</i>	15.7±3.2 ^{B,C}	23.7±2.5 ^{b,B}	28.7±0.6 ^{A,a}
<i>Pseudomonas aeruginosa</i>	15.3±4.5	21.5±2.1 ^b	25.7±3.8 ^a
<i>Pseudomonas fluorescens</i>	65.0±0.0 ^{**}	65.0±0.0 ^{**}	65.0±0.0 ^{**}
<i>Pseudomonas putida</i>	65.0±0.0 ^{**}	65.0±0.0 ^{**}	65.0±0.0 ^{**}
<i>Listeria innocua</i>	9.0±1.4	12.5±0.7	17.0±7.1
<i>Listeria monocytogenes</i>	9.3±2.1 ^b	16.3±3.2 ^a	15.3±1.5 ^a
<i>Staphylococcus aureus</i>	11.7±2.1 ^B	15.0±2.0 ^B	24.7±3.5 ^A

Table 2

Mean rays of inhibition halos produced by *L. animalis* SB310, *L. paracasei* subsp. *paracasei* SB137 and the mixture of them grown together against spoilage or pathogenic target microorganisms.

A, B, C ($P < 0.01$); a, b, c ($P < 0.05$). Values from different target strains were statistically considered separately.

*1:1a: mixture prepared adding the two strains before the incubation and grown together.

** : halo > 65 mm, maximum detectable halo.

4.4.2 Trial 2 - Antimicrobial activity of different mixtures of viable *Lactobacilli*

The mean rays of the inhibition halos obtained in the second trial are reported in Table 3. The halos measured in trial 2, regarding *L. animalis* SB310 and *L. paracasei* subsp. *paracasei* SB317, were slightly higher than those obtained from trial 1, generally due to a higher acidification of the culture used in trial 2 (e.g. pH *L. paracasei* trial 1= 3.79, pH *L. paracasei* trial 2=3.61). These differences were not statistically significant with the only exception of *L. paracasei* against *L. monocytogenes* and *L. innocua*, due to their low pH growth limit and ability to adapt to acid environment. According with the preliminary test, the individual *Lactobacillus* strains and the mixtures confirmed to exert an inhibitory activity against all the bacteria tested (96.3% of all the halos detected were halos were >10 mm). Moreover, 44.4% of the plates inoculated with the mixtures, independently from the percentage of composition of the two strains, showed a halo > 20 mm.

In most of cases, the effect of the combination of the two strains, even if dependent on the typology of mixture, was greater than those exerted by the individual *Lactobacilli*. *L. animalis* always produced significantly lower halos than the mixtures and *L. paracasei* ($P < 0.0001$). Moreover, the mixture containing the highest rate of *L. animalis* (A10) produced significantly lower halos ($P < 0.05$) if

compared to the mixtures 1:1b, 1:5, 1:10 and to *L. paracasei* (P), confirming the higher ability to acidify of *L. paracasei* subsp. *paracasei*, according to Ripamonti et al. (2011). Even if *L. paracasei* showed lower rays than almost all of the mixtures with higher composition of this strains (P10, P5), no statistically significant differences were detected. Also in this trial, for *P. fluorescens* and *P. putida* no growth was detected within the plates for all the cultures tested and an arranged value of 65 mm was assigned. Among the other target species, *Y. enterocolitica* resulted as the most susceptible strain showing significantly wider halos ($P < 0.001$) than all the other strains except for *L. innocua* and *P. aeruginosa*. All the mixtures tested against Gram positive bacteria and *P. aeruginosa* showed very similar values, without an evident trend with the increasing concentration of *L. paracasei* in the mixtures. In particular, *S. marcescens* and *E. coli* O157:H7, as stated by trial 1, resulted as poorly susceptible strains producing very limited halos; *S. marcescens* was the only strain showing limited halos (< 15 mm) as far as the rate of *L. paracasei* increased in the 2:1 mixture.

In any case, the combination of the two strains with a prevalence of *L. paracasei* subsp. *paracasei* in the composition resulted as more effective than the other mixtures, confirming the great potentiality and synergism of the two strains, if applied together. In the light of the results acquired from the first and the second trial, the effect of the mixture 1:1a (obtained adding the two *Lactobacillus* strains with similar OD before the incubation) could be related to a better growth of *L. paracasei* subsp. *paracasei* SB317 which we could suppose to be able to replicate faster than *L. animalis* SB310 (confirmed even by the higher OD regularly detected before the preparation of the mixtures and by the necessity to dilute this strain). Consequently, the lower effect detected by the 1:1b mixture (obtained adding the strains after incubation and after OD adjustment if compared with 1:1a mixture) was basically due to the dilution of *L. paracasei*.

Target strains	A	10:1	5:1	2:1	1:1b	1:2	1:5	1:10	P
<i>Escherichia coli</i>	11.0 ±4.2 ^{B,b}	18.0 ±2.1 ^A	17.0 ±1.4 ^a	17.5 ±1.4 ^A	19.5 ±0.0 ^A	17.0 ±0.7 ^a	19.5 ±0.7 ^A	19.0 ±1.4 ^A	17.5 ±0.7 ^A
<i>E. coli</i> O157:H7	8.0 ±1.4 ^{B,b}	15.5 ±3.5 ^a	15.5 ±2.1 ^a	15.0 ±0.7	16.5 ±0.7 ^a	16.5 ±0.7 ^a	22.0 ±1.4 ^A	17.0 ±1.4 ^a	17.5 ±2.1 ^a
<i>Protens vulgaris</i>	15.0 ±1.4 ^{B,b}	17.5 ±1.4 ^{a,B,b}	22.0 ±0.7 ^{A,b}	23.5 ±4.2 ^{A,a}	20.5 ±1.4 ^{A,a,b,B}	21.0 ±2.1 ^{A,b,B}	20.5 ±0.7 ^{A,a,b,B}	25.0 ±2.1 ^{A,a}	24.0 ±0.7 ^{A,a}
<i>Salmonella Typhimurium</i>	16.5 ±0.7 ^{B,b}	17.5 ±0.0 ^b	19.0 ±0.7	18.0 ±0.7 ^b	17.5 ±0.0 ^b	21.5 ±0.7 ^{A,a}	21.0 ±0.0 ^a	17.5 ±2.1 ^b	20.0 ±3.5
<i>Serratia marcescens</i>	10.0 ±0.0	12.5 ±2.8	12.5 ±2.1	16.0 ±4.2	16.0 ±6.4	17.0 ±7.8	15.5 ±4.2	18.0 ±3.5	17.0 ±2.8
<i>Yersinia enterocolitica</i>	15.5 ±3.5	18.5 ±4.2	29.0 ±16.3	28.0 ±0.0	26.5 ±1.4	25.0 ±0.7	22.5 ±1.4	27.5 ±1.4	25.5 ±0.7
<i>Pseudomonas aeruginosa</i>	10.5 ±6.4 ^b	26.5 ±9.2 ^a	25.5 ±2.1 ^a	23.5 ±1.4 ^a	25.0 ±3.5 ^a	21.0 ±4.2	26.5 ±2.1 ^a	22.5 ±0.0	26.0 ±0.0 ^a
<i>Pseudomonas fluorescens</i>	65.0 ±0.0 [*]	65.0 ±0.0 [*]	65.0 ±0.0 [*]	65.0 ±0.0 [*]	65.0 ±0.0 [*]	65.0 ±0.0 [*]	65.0 ±0.0 [*]	65.0 ±0.0 [*]	65.0 ±0.0 [*]
<i>Pseudomonas putida</i>	65.0 ±0.0 [*]	65.0 ±0.0 [*]	65.0 ±0.0 [*]	65.0 ±0.0 [*]	65.0 ±0.0 [*]	65.0 ±0.0 [*]	65.0 ±0.0 [*]	65.0 ±0.0 [*]	65.0 ±0.0 [*]
<i>Listeria innocua</i>	10.5 ±0.7	22.5 ±6.4	22.5 ±3.5	24.5 ±2.1	22.5 ±0.7	25.5 ±0.7	26.5 ±0.7	25.0 ±2.1	24.5 ±0.7
<i>Listeria monocytogenes</i>	13.5 ±2.1 ^B	18.0 ±0.7 ^{A,b}	20.5 ±2.8 ^{A,b}	24.0 ±0.0 ^A	23.0 ±0.7 ^{A,b}	22.0 ±0.7 ^A	22.0 ±2.8 ^{A,a,b}	23.0 ±0.7 ^A	23.0 ±2.8 ^A
<i>Staphylococcus aureus</i>	13.0 ±2.8 ^B	21.0 ±3.5 ^{A,a}	18.5 ±1.4 ^A	19.0 ±5.7 ^{A,b}	19.0 ±6.4 ^{A,b}	18.5 ±2.1 ^A	20.5 ±1.4 ^A	20.0 ±4.2 ^{A,b}	16.5 ±7.1 ^{A,b}

Table 3

Mean rays of inhibition halos produced by *L. animalis* SB310, *L. paracasei* subsp. *paracasei* SB137 and different mixtures of them against spoilage or pathogenic target microorganisms.

^{A, B} ($P < 0.01$); ^{a, b} ($P < 0.05$). Values from different target strains were statistically considered separately.

*: halo > 65 mm, maximum detectable halo.

The abbreviations reported are explained in Table 1.

4.4.3 Antimicrobial activity of cell-free supernatants of single strains and the mixtures (1:1a, 1:1b, 1:2, 1:5, 1:10, 2:1, 5:1, 10:1)

In table 4 are reported the results from the susceptibility test of the cell-free supernatants of the single strains and of the mixtures 1:1a (trial 1). A very limited activity was recorded for all the 12 strains tested against the single strains and all the mixtures if compared with the action of viable Lactobacilli. This highlights that the antimicrobial effect originates from a combination of bacterial competition and the production of extracellular compounds. No correlation was revealed between the data obtained from viable cells and cell-free supernatants. *Pseudomonas* spp. confirmed their higher sensitivity; however contrasting results were obtained for the other microorganisms. For example, for *Serratia marcescens* wider halos were produced by the supernatants compared to the other species, while *Staphylococcus aureus* was the most resistant strain tested. No statistically significant differences were detected among the 12 bacteria tested. Considering

Lactobacillus strains, no statistically significant differences were observed among the single strains and all the mixtures tested. In order to clarify the nature of antimicrobial compounds, pH values of the supernatants were registered; after incubation, an evident acidification of the culture broth was observed, with *L. paracasei* being the most active. In trial 1 the pH value of 3.98 was detected in *L. animalis* supernatant, while a pH value of 3.79 was detected in *L. paracasei*.

In trial 2 no significant inhibition halos were recorded (values ranging from 0.2 to 0.6 mm), without any difference among the target species and different mixtures, confirming the importance of the presence of viable Lactobacilli and the production of extracellular compounds to exert an efficient antimicrobial.

In the same trial, a pH value of 3.74 was detected in *L. animalis* supernatant, and a gradual decrease was observed as the *L. paracasei* rate increased, till a value of 3.61 (100% *L. paracasei* supernatant), evidencing lower pH values in *L. paracasei* if compared with trial 1. The coculture grown together (mixture 1:1a) showed a pH value equal to *L. paracasei* subsp. *paracasei*, suggesting a higher growth rate of this microorganism. This different acidifying activity was expected, as a previous study (Ripamonti et al., 2011) reported the production of lactic and acetic acids by these two strains, with a higher production of acetic acid by *L. paracasei* subsp. *paracasei*.

Several authors have shown the inhibition of a broad range of microorganisms and demonstrated the importance of these two weak organic acids for the antimicrobial efficacy of *Lactobacillus* strains. The synergism between the two molecules is due to the acidification of lactic acid which favors the presence of the undissociated form of acetic acid, thus enhancing its antimicrobial activity (Adams & Hall, 1988; Helander, von Wright & Mattila-Sandholm, 1997; Castellano, Belfiore, Fadda, & Vignolo, 2008). The importance of the production of organic acids was confirmed by the absence of antimicrobial activity by the cell-free supernatants adjusted to pH 6.5, suggesting the absence of other inhibitory compounds, such as bacteriocins or hydrogen peroxide.

Target strains	<i>L. animalis</i> SB310	<i>L. paracasei</i> subsp. <i>paracasei</i> SB137	Mixture 1:1a
<i>Escherichia coli</i>	0.6±0.3	0.7±0.6	0.5±0.4
<i>Escherichia coli</i> O157:H7	0.5±0.3	0.4±0.5	0.8±0.7
<i>Proteus vulgaris</i>	0.7±0.5	0.8±0.3	0.9±0.4
<i>Salmonella</i> Typhimurium	0.6±0.5	0.5±0.6	0.3±0.3
<i>Serratia marcescens</i>	1.4±1.0	1.0±0.4	1.0±0.5
<i>Yersinia enterocolitica</i>	1.0±0.2	1.2±0.7	0.9±0.5
<i>Pseudomonas aeruginosa</i>	0.9±0.4	1.7±0.9	1.2±0.4
<i>Pseudomonas fluorescens</i>	1.2±0.8	1.6±0.8	0.8±0.5
<i>Pseudomonas putida</i>	1.3±1.0	1.7±0.7	1.3±0.6
<i>Listeria innocua</i>	0.5±0.7	0.5±0.6	0.8±0.6
<i>Listeria monocytogenes</i>	0.6±0.6	1.1±1.4	0.7±0.9
<i>Staphylococcus aureus</i>	0.2±0.1	0.3±0.4	0.8±0.4

Table 4

Halos, expressed as a mean of six replications, induced by the cell-free supernatant of *L. animalis* SB310, *L. paracasei* subsp. *paracasei* SB137 and their mixture (1:1a) against spoilage or pathogenic target microorganisms (trial 1).

4.5 Conclusions

Historically lactobacilli have been recognized for their useful role in food preservation by inhibiting the growth of spoilage and pathogenic microorganisms without the production of sensorial changes. *Lactobacillus animalis* SB310 and *L. paracasei* subsp. *paracasei* SB137 tested in this work have never been studied before for this purpose and there is limited knowledge in the literature regarding their bioprotective properties.

Our results showed the promising antimicrobial activity of the two strains and in particular of the mixture of the two (especially when *L. paracasei* subsp. *paracasei* SB137 was predominant) against a wide number of spoilage and pathogenic bacteria, due mainly to the production of organic acids (acetic and lactic) as a combined effect. Further studies are necessary to clarify the effect of the inoculation of these strains in meat, which is characterized by a complex chemical environment.

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4.7 References

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CHAPTER 5

Third study:

Evaluation of the *in vitro* antimicrobial activity of mixtures of *Lactobacillus sakei* and *L. curvatus* isolated from Argentine meat and their application on vacuum-packed beef

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5. Evaluation of the *in vitro* antimicrobial activity of mixtures of *Lactobacillus sakei* and *L. curvatus* isolated from Argentine meat and their application on vacuum-packed beef

AUTHORS: *Simone Stella, Cristian Bernardi, Patrizia Cattaneo, Fabio Colombo, Erica Tirloni*

Department of Health, Animal Science and Food Safety, Università degli Studi di Milano, via Celoria 10, 20133, Milano, Italy

5.1 Abstract

Two specific mixtures (one *L. sakei*-based mixture and one *L. curvatus*-based mixture), obtained adding strains isolated from long shelf life vacuum-packaged beef from Argentina, their cell-free supernatants and buffered cell-free supernatants were tested *in vitro* against a wide range of spoilage or potentially pathogenic bacteria. In almost all the cases, the inhibition halos produced by *L. curvatus* mixture were significantly higher than those produced by *L. sakei* one. Among the target bacteria, *Pseudomonas fluorescens* was clearly the most susceptible; a high variability in *Enterobacteriaceae* was detected, identifying *Serratia marcescens* as the less susceptible strain. No activity was exerted by the cell-free supernatants, for all the target strains tested, highlighting that the antagonistic effect originates probably from a combination of nutrient competitive exclusion and a lowering of the pH. Moreover, the effect of the addition of the two mixtures to vacuum-packaged beef slices was investigated, considering microbiological and physico-chemical parameters. No significant effects of LAB mixtures on meat pH and colour parameters were detected. Lower loads of Total Viable Count, *Enterobacteriaceae* and Gram negative bacteria were generally observed in inoculated samples, if compared with the control ones. However, the addition of the mixtures resulted to be less effective if applied to meat substrate, as high bacterial loads were detected. This could be explained by the different competitiveness of the cultures if applied to a complex substrate like meat and to the limited acidification due to the buffering capacity of meat.

5.2 Introduction

Meat preservation is a hard race against spoilage and potential pathogenic microorganisms and restriction methods need to be applied in order to reduce their growth and prolong the shelf life. Recently, alternative technologies for the decontamination of meat products have been developed and implemented such as bioprotective cultures, natural antimicrobials, gamma, electron and x-ray irradiation, ozone, active packaging, high hydrostatic pressure, ohmic heating and steam pasteurization among the others (Loretz and others 2011; Zhou and others 2010; Aymerich and others 2008; Devlieghere and others 2004). All the alternative technologies effort to be mild: their combination, as in the hurdle theory proposed by Leistner (2000), may improve their efficacy against pathogens and spoilage microorganisms, without modifying the sensorial qualities of the products.

In chilled vacuum-packaged raw meat, the oxygen source is restricted determining a selective effect on the microbial population; the main spoilage microorganisms associated with these type of food results as psychrotrophic, both Gram-positive bacteria, mainly Lactic Acid Bacteria (LAB) (*Lactobacillus* spp., *Leuconostoc* spp., *Carnobacterium* spp.) and *Brochothrix thermosphacta*, and Gram-negative, mainly represented by *Enterobacteriaceae* (Pennacchia and others 2011; Ercolini and others 2009; Fontana and others 2006; Nychas and Drosinos 2000; Labadie 1999; Holzapfel 1998; Shaw and Harding 1984). In vacuum packaged meat, the natural LAB population increases during storage, becoming the predominant microflora: in particular, at chilling temperatures, LAB are able to exert antagonistic actions towards the growth of spoilage and pathogenic microorganisms in beef, pork, poultry and fish (Castellano and others 2008; Katla and others 2002; Yamazaki and others 2003).

In the last years, LAB have received great consideration as bioprotective cultures, leading to the discovery and characterization of several antimicrobial peptides (mainly bacteriocins, organic acids, carbon dioxide, ethanol, hydrogen peroxide and diacetyl), whose antimicrobial activity is well known (Aymerich and others 2008; Dortu and others 2008; Ravyts and others 2008; Castellano and Vignolo 2006; Cleveland and others 2001; Vignolo and others 2000). Their action is also due to the lowering of food pH and to the competition for nutrients (Vandenbergh 1993).

Different studies indicated that, during the storage, a gradual selection of LAB species occurs in the meat ecosystems, leading to the predominance of few *Lactobacillus* species (Vignolo and others 2012; Vignolo and others 2010); *L. sakei* and *L. curvatus* have been observed as the most widespread species in vacuum-packaged beef (Stella and others 2013; Fontana and others 2006; Yost and Nattress 2002).

Previous studies underlined the abilities of these two species as bioprotective cultures for meat, and their application to vacuum-packaged Argentine beef has already been described (Castellano and Vignolo 2006; Vignolo and others 2010). Their mechanism of action is expressed not only through the ability to produce bacteriocins but even organic acids. Moreover the good adaptation to meat environment of *L. curvatus* and *L. sakei* was already proved, showing an important competitiveness in this substrate and an efficient use as an extra hurdle to minimize the risk of listeriosis in different muscle foods (Fadda and others 2008; Castellano and Vignolo 2006; Hugas 1998; Schillinger and others 1991).

In a previous work 73 Lactobacilli were isolated from 8 lots of vacuum-packaged bovine rump hearts imported in Italy from Argentina, submitted to random amplified DNA-polymerase chain reaction and identified, showing a prevalence of *Lactobacillus sakei* (56 isolates grouped in 18 different clusters) and *Lactobacillus curvatus* (8 isolates grouped in 6 different clusters) (Stella and others 2013).

One strain from each of the most representative clusters obtained of *L. sakei* (≥ 5 strains) and *L. curvatus* (≥ 2 strains), for a total 6 *L. sakei* and 2 *L. curvatus* isolates, of were chosen. Two specific mixtures were prepared (one *L. sakei*-based mixture and one *L. curvatus*-based mixture) and evaluated *in vitro* for their antimicrobial activity against spoilage and potential pathogenic microorganisms. Moreover, the effect of the addition of the two mixtures to sliced vacuum-packaged beef was investigated, considering microbiological and physical-chemical parameters.

5.3 Materials and methods

5.3.1 Preparation of *Lactobacillus* strains

All *L. sakei* and *L. curvatus* strains were stored in cryovials (Microbank™, Pro-Lab Diagnostics, Richmond Hill, Canada) at -70°C until the use. For each strain, a loop of the frozen culture was transferred to a test tube containing 10 mL of MRS broth (Oxoid, Basingstoke, UK) and incubated overnight at 30°C in jars (Anaerojar, Oxoid) with anaerobiosis generators (AnaeroGen, Oxoid). All the strains were re-inoculated into cooled MRS broth tubes and the initial absorbance (540 nm) (Shimadzu, UV1601, McCormick Place, Chicago, IL, USA) was measured. All the tubes were incubated at 15°C and the absorbance was measured after 24 and 48 h. Precultures were collected in exponential growth rate, defined as a change of absorbance of 0.05-0.2 at 540 nm. If necessary, the cultures were diluted before preparing the mixture in order to obtain the similar OD. Two specific mixtures were prepared (*L. sakei*-based mixture of isolates n° 3, 42, 55, 77, 106 and 111 and *L. curvatus*-based mixture of isolates n° 25 and 65) adding the same aliquot of broth of each strain.

5.3.2 *Antimicrobial activity against spoilage and potential pathogenic microorganisms*

For the evaluation of the antimicrobial activity, each mixture, prepared as reported above, was inoculated into MRS broth tubes and incubated at 30°C for 48 h in anaerobiosis. After incubation, each of the two broths was spotted by a sterile swab (Carlo Erba, Rodano, I) onto the surface of MRS agar plates, subsequently incubated for 48 h at 30°C in an anaerobic jar. A selection of 12 spoilage or pathogenic microorganisms was used as target for the test: *Escherichia coli* ATCC 25922, *Escherichia coli* 0157:H7 DSM 13526, *Proteus vulgaris* ATCC 8427, *Salmonella* Typhimurium ATCC 14028, *Serratia marcescens* ATCC 14756, *Yersinia enterocolitica* ATCC 23715, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas fluorescens* ATCC 13525, *Pseudomonas putida* ATCC 49128, *Listeria monocytogenes* ATCC 7644, *Listeria innocua* ATCC 33090 and *Staphylococcus aureus* ATCC 6538. Each strain, stored in cryovials at -70°C until the use, was subcultured aerobically overnight at 37°C (30°C for *P. fluorescens* and *P. putida*) in 10 mL TSB tubes (Tryptic Soy Broth, Oxoid). All the strains were re-inoculated into cooled TSB tubes and the initial absorbance was detected. All the tubes were incubated at 15°C and the absorbance was measured after 24 and 48 h. Precultures were collected in exponential growth rate, defined as a change of absorbance of 0.05 at 540 nm. If necessary, the cultures were diluted before the test in order to obtain a similar OD. For each spoilage or pathogenic microorganism, 0.2 mL of bacterial suspension were added to a 5 mL share of semisolid agar (BHI, Brain Heart Infusion Broth, Oxoid + agar 0.7%), maintained in a water bath (45°C) and then poured over the MRS plates previously spotted with each mixture. To avoid the dispersion of *Lactobacilli* from the spot into BHI, a little amount (3-4 drops) of the inoculated semisolid medium was firstly distributed by a sterile Pasteur pipette (Carlo Erba) on the surface of the spot; after solidification (about 3 minutes at room temperature), the remaining BHI was poured on the plates. After aerobic incubation at 37°C (30°C for *P. fluorescens* and *P. putida*) for 24 h, the plates were checked. A clear zone around the *Lactobacillus* spot indicated the inhibition of the target microorganisms. The tests were conducted in triplicate.

5.3.3 *Antimicrobial activity of cell-free supernatants against spoilage and potential pathogenic microorganisms*

In order to determine if the inhibition was due to the production of antagonistic compounds, the cell-free supernatants of the cultured mixtures were tested against the same bacteria. The mixtures were subcultured in MRS broth as described above. After 48 h of incubation, an aliquot of each culture was centrifuged at 7700 rpm for 10 min. For each broth, pH was measured by a pH meter (Ghiaroni, XS pH6, Buccinasco, I): three independent measurements were

performed on each sample. The supernatants obtained were subsequently filtered by 0.2 μm filters (Sacco, Cadorago, I) and maintained at 4°C. Each of the 12 target strains were inoculated into 10 mL TSB tubes and prepared as described in section 2.2; 1 mL of inoculated TSB was then transferred into 20 mL flasks of Tryptic Soy Agar (Oxoid), maintained in a water bath at 45°C, carefully mixed and poured in sterile Petri plates. Once the media were solidified, blank discs (Oxoid) were dipped with the supernatant of each mixture and placed onto the plates, subsequently incubated at 37°C (30°C for *P. fluorescens* and *P. putida*) for 24 h. Clear zones around the discs were recorded. Finally, in order to evaluate if the eventual inhibition was due to the production of organic acids, the pH of cell-free supernatants were adjusted to 6.5 with NaOH (1 N) (Sigma, Milano, I) and the same test was repeated. All the tests were performed in triplicate.

5.3.4 Preparation and inoculation of vacuum-packaged meat slices

Two bovine rump hearts were sliced at a commercial cutting plant. From each meat cut, a total of 42 slices (1-cm thick, 50 g of weight) were obtained and inserted into individual sterile plastic bags, with a diffusion coefficient of 6/14 $\text{cm}^3 \text{m}^{-2} \text{atm}^{-1} 24 \text{h}^{-1}$ to oxygen at 25°C and 75% relative humidity (Cryovac, Elmwood Park, NJ). The 42 slices obtained from each rump heart were grouped into two series (each series including 21 discs) inoculated as follows:

- CLS (Control samples *L. sakei*), inoculated with 0.5 mL of sterile saline solution;
 - LS (*L. sakei*), inoculated with 0.5 mL of a mixture of the six strains of *L. sakei* (final concentration of 5 Log CFU/g);
- and:
- CLC (Control samples *L. curvatus*), inoculated with 0.5 mL of sterile saline solution;
 - LC (*L. curvatus*), inoculated with 0.5 mL of a mixture of the two strains of *L. curvatus* (final concentration of 5 Log CFU/g).

A loop of the frozen culture of each strain was transferred to a test tube containing 10 mL of MRS broth (Oxoid) and incubated overnight at 30°C in jars (Anaerojar, Oxoid) with anaerobiosis generators (AnaeroGen, Oxoid). All the strains were re-inoculated into cooled MRS broth tubes and the initial absorbance (540 nm) was detected. All the tubes were incubated at 15°C and the absorbance was measured after 24 and 48 h. Precultures were collected in exponential growth rate. The bacterial cells were pelleted by centrifugation at 7700 rpm for 10 min at 4°C and washed twice in 10 mL of 0.1 M phosphate buffered saline (PBS) with pH 7.0. Cell density of each strain was determined by microscopy (100x) (Meiji Techno America, USA). An average value from 10

randomly picked fields of view was considered. As needed, precultures were diluted in 0.85% NaCl solution prior to inoculate the products and *L. sakei*-based mixture of isolates n° 3, 42, 55, 77, 106 and 111 and *L. curvatus*-based mixture of isolates n° 25 and 65 were finally prepared adding the same aliquot of each strain at a final concentration nearly of 5 Log CFU/mL.

After inoculation, the plastic bags were submitted to a vacuum pump (final vacuum of 99%), sealed using a packaging machine (Orved VM 16, Musile di Piave, I) and immediately stored at 4°C. Samples were submitted in triplicate to analyses after inoculation (T0) and after 10 (T10), 20 (T20), 30 (T30), 40 (T40), 50 (T50) and 60 (T60) days of storage.

5.3.5 Microbiological analyses

10 g of each sample were diluted in physiological saline (0.85% NaCl) with 0.1% peptone and homogenized in a Stomacher for 60 s (Seward Stomacher 400 Blender Mixer Homogenizer, International PBI, Milano, IT). Serial 10-fold dilutions were prepared and the following parameters were evaluated: Total Viable Count (TVC) was performed on Plate Count Agar (PCA, Biogenetics, Ponte San Nicolò, I) (ISO 4833:2003) and incubated at 30°C for 48h; Lactobacilli were enumerated on MRS agar (Oxoid) (ISO 15214:1998) incubated at 30°C for 48h in anaerobiosis, Gram negative bacteria were enumerated on Tryptone Soy Agar (Oxoid) supplemented with 10 UI/mL of penicillin G (Oxoid) (TSAP) and incubated at 30°C for 48h; the number of *Enterobacteriaceae* was determined on Violet Red Bile Glucose Agar (VRBGA, Biogenetics) according to the ISO 21528-2:2004 method.

5.3.6 Physical and chemical analyses

At each sampling time, pH was measured by a pH meter: three independent measurements were performed on each sample and means were calculated.

The surface colour of the meat was assessed 45 min after opening the packages, in order to allow blooming (deoxymyoglobin oxygenation) on six randomly chosen spots of each sample surface using a Minolta CR-200 Chromameter (Minolta, Osaka, J). L^* (lightness), a^* (“red” index) and b^* (“yellow” index) parameters were determined; Hue angle was also calculated as $\arctan(b^*/a^*) \cdot 57.29$.

5.3.7 Statistical analysis

The experimental data from inhibition halos were analyzed by a two-way univariate analysis of variance (SAS Inst. Inc., Cary, NC, 2006) in order to

compare the activity of the two *Lactobacillus* mixtures. Differences in inhibition halos among the target strains within each *Lactobacillus* mixture were also checked. Data from meat inoculation tests were also analyzed by a two-way univariate analysis of variance to reveal the difference between treated samples and the respective control ones. For all statistical evaluations, threshold levels of $P \leq 0.05$ and $P \leq 0.01$ were considered for significance.

5.4 Results and discussion

5.4.1 Antimicrobial activity against spoilage and potential pathogenic microorganisms

The mean rays of the inhibition halos obtained from antimicrobial evaluation of *L. sakei* mixture and *L. curvatus* mixture are reported in table 1. The two mixtures exerted an antimicrobial activity, producing evident halos against all the 12 target strains tested (66.7% of the halos induced by *L. curvatus* mixture and 52.8% of halos produced by *L. sakei* mixture were >10 mm). Generally, *L. curvatus* mixture resulted significantly more effective if compared to *L. sakei* mixture ($P=0.0383$), showing also a higher prevalence of halos > 20 mm (19.4% of the plates inoculated with the *L. curvatus* mixture vs 5.5% of those inoculated with *L. sakei* mixture). Considering the different target strains, *L. curvatus* mixture produced significantly wider halos than *L. sakei* against *Y. enterocolitica* ($P=0.0383$) and *P. aeruginosa* ($P=0.0325$) and generally produced higher halos towards almost all the bacteria tested, except for *E. coli*, *L. innocua* and *S. marcescens*.

If we consider the results of the target strains tested clustered in homogenous categories, it is evident that the most sensitive resulted to be the *Pseudomonas* spp., whose components produced significantly higher halos if compared with *Enterobacteriaceae* ($P<0.0001$), *Listeria* spp. ($P=0.0004$) and *Staphylococcus aureus* ($P=0.0117$). As a matter of fact, for both the two mixtures tested, the most sensitive strains resulted *Pseudomonas* spp., according to Tirloni et al. (2014) and Moore and others (2006) who underlined that most of the species of *Pseudomonas* fail to grow under acid conditions.

P. fluorescens resulted to be the most susceptible among the 12 target strains tested as significantly wider halos were observed if compared with all the other strains ($P<0.01$). Secondly, *P. putida* resulted to be significantly more susceptible if compared to *E. coli* O157:H7 ($P=0.0357$), *E. coli* ($P=0.0215$), *L. innocua* ($P=0.0200$), *L. monocytogenes* ($P=0.0411$), *P. vulgaris* ($P=0.0084$), *S. marcescens* ($P=0.0003$) and *S. Typhimurium* ($P=0.0215$). Finally *P. aeruginosa* produced significantly wider halos if compared to *S. marcescens* ($P= 0.0185$).

Moreover, *Enterobacteriaceae* showed a high variability in susceptibility with differences among the various species due to the many interspecific and intraspecific differences among the bacteria tested (Liu and others 2013). *Serratia marcescens* was by far the most resistant target strain, and it showed significantly

smaller halos if compared to *Y. enterocolitica* (P=0.0116), the most sensitive of *Enterobacteriaceae*.

Many authors highlighted the presence of an evident antagonistic activity of LAB against *Listeria monocytogenes*, microorganism typically related to vacuum-packaged meat products (Awaishah and Ibrahim 2009; Jones and others 2008). Even in our study, both *L. monocytogenes* and *L. innocua*, showed the production of modest halos (between 9.7 and 14 mm).

Considering the cell-free supernatants and the pH-adjusted supernatants, no activity was recorded for all the target strains tested, highlighting that the antagonistic effect originates probably from the nutrient competitive exclusion while the involvement of extracellular compounds was not detected for the species considered in this test. The mechanism of the antibacterial activity of *Lactobacillus* strains usually appears to be multifactorial: the well-known production of bacteriocins by *L. sakei* and *L. curvatus* strains, reported in many previous studies (Castellano and others 2010; Castellano and others 2008; Castellano and Vignolo 2006) was not confirmed in our research. Furthermore, in this case, we could suppose that the antagonistic activity detected was also related to the production of metabolites such as organic acids, determining a lowering of the pH; in our samples, the mixtures tested determined a pH decrease from 6.40 to 4.16 (LC) and 4.19 (LS). Nevertheless, in order to obtain an important inhibition of the target strains growth, the presence of live and metabolically active live cells, is fundamental.

Target strains	<i>L. curvatus</i> mixture (mm)	<i>L. sakei</i> mixture (mm)
<i>Escherichia coli</i>	8.3±1.5	13.3±3.8
<i>Escherichia coli</i> O157:H7	14.0±2.6	10.0±2.0
<i>Proteus vulgaris</i>	10.3±1.2	7.3±1.5
<i>Salmonella</i> Typhimurium	13.3±4.9	8.3±2.9
<i>Serratia marcescens</i>	2.3±1.2	2.7±1.2
<i>Yersinia enterocolitica</i>	19.7±3.1 ^a	13.7±1.5 ^b
<i>Pseudomonas aeruginosa</i>	21.3±3.5 ^a	10.0±5.0 ^b
<i>Pseudomonas fluorescens</i>	56.7±23.1	21.7±13.5
<i>Pseudomonas putida</i>	30.0±34.7	17.3±2.5
<i>Listeria monocytogenes</i>	14.0±3.6	10.7±1.5
<i>Listeria innocua</i>	11.7±5.7	9.7±2.3
<i>Staphylococcus aureus</i>	13.7±1.5	12.7±1.5

Table 1

Halos expressed as a mean of three replication induced by *L. curvatus* mixture and *L. sakei* mixture against spoilage or pathogenic target microorganisms. ^{a,b} (P < 0.05).

5.4.2 Inoculation of vacuum-packaged meat slices

LAB cultures, and in particular *L. sakei* and *L. curvatus*, have been often studied for the application to food with good results thanks to the inhibition of pathogens and spoilage microorganisms and with the aim to extend the shelf-life of raw meat without important changes in the sensory properties of the product (Castellano and Vignolo 2006).

Considering the global effect of the application of *L. sakei* mixture to meat during the whole trial, TVC resulted significantly lower in samples treated with the mixture (LS) if compared with the control ones (CLS) ($P=0.0089$), reaching at the end of the trial the loads of 5.8 ± 0.4 and 7.4 ± 0.5 Log CFU/g, respectively. The addition of *L. sakei* mixture resulted, since the beginning of the trial, in a constant higher level of LAB in LS samples if compared with CLS ($P<0.0001$). In particular, in LS samples, LAB reached the plateau level between 8 and 8.5 Log CFU/g after only 20 days from the beginning of the experiment. In CLS samples, the LAB naturally present on the slices showed a rapid increase from the beginning until T20; afterwards, they reached a plateau level between 6.6 and 7.4 Log CFU/g. Considering Gram negative bacteria for the whole period, LS samples values resulted to be significantly lower than CLS ones ($P=0.0029$). Moreover, *Enterobacteriaceae* resulted to be significantly lower in LS samples considering the whole trial ($P<0.0001$). In particular in LS samples they showed a very stable trend (LS T0= 2.3 ± 0.5 vs T60= 2.3 ± 0.5 Log CFU/g), while in CLS samples, *Enterobacteriaceae* reached a value of 4.4 ± 1.9 Log CFU/g at T60; anyway such level of contamination is not generally associated to evident sensorial spoilage of raw meats.

Considering the effect obtained from the application of *L. curvatus* mixture to meat in the whole experimental period, TVC resulted significantly lower in treated samples (LC) than in control (CLC) ones ($P=0.0013$). The addition of *L. curvatus* mixture resulted, since the beginning of the trial, in a constant higher level of LAB in LC samples if compared with CLC ($P<0.0001$). In particular, in LC samples, LAB reached the plateau level between 7.9 and 8.4 Log CFU/g after only 20 days from the beginning of the experiment, according with LS results. In CLC samples, the LAB naturally present in the product, characterized in this case by a higher load if compared with CLS (3.5 ± 0.6 Log CFU/g at T0), showed a rapid increase from T20; afterwards, they reached a plateau level between 6.1 and 7.5 Log CFU/g.

Considering Gram negative bacteria, the loads resulted to be quite comparable between LC and CLC samples until T20 and then very highly fluctuant data were obtained; considering the whole period no statistical significance was recorded ($P=0.3325$).

Enterobacteriaceae resulted to be constantly lower in LC samples until T60, showing a general significant difference ($P=0.0225$): in particular they showed a

very stable trend for the whole study (LC T₀=2.3±0.6 *vs* T₆₀=3.0±1.5 Log CFU/g). In CLC samples, *Enterobacteriaceae* showed an increasing trend since the beginning of the trial, even if not reaching the threshold level of 5 Log CFU/g (CLS T₀=2.5±0.8 *vs* T₆₀=4.0±1.8 Log CFU/g).

The effect of the inoculation with *L. sakei* mixture resulted generally more evident than the treatment with *L. curvatus* mixture, suggesting a better capability to adapt to vacuum packaged meat substrate. The better adaptation of *L. sakei* mixture (LS) confirmed the preponderance of *L. sakei* in long shelf-life vacuum packaged meat LAB population, as highlighted in the previous study (Stella and others 2013).

In any case, the capability of both of the two LAB mixtures to inhibit the growth of spoilage bacteria, clearly demonstrated *in vitro*, was also confirmed on meat substrate, also if it resulted more limited. This could be explained by the different growth rates and competitiveness of the cultures if applied to a complex matrix like meat: the adaptation to a substrate depends especially on the metabolic activity of cultures, which occupy vital niches, thus discouraging colonisation of undesired microorganisms. Generally, an antagonistic effect was detected both for *L. sakei* and *L. curvatus* treatments, even if TVC and Gram negative bacteria were characterized by high loads. Despite the promising current knowledge and laboratory studies, LAB strains often suffer from a limited effectiveness in foods: among the others, the main factors involved are the poor adaptation to food environment, the inactivation of antimicrobial compounds through proteolytic enzymes or the binding to food ingredients and the pH buffering action (Holzapfel and others 1995). In our case, the production of organic acids by the cultures inoculated on meat is supposable, even if their activity could be limited by the buffering capacity of meat. The metabolic activity of LAB population of vacuum packed meat could be deduced by the slight reduction of pH of meat during the trial, with a 0.25 and 0.39 decrease in meat treated with the two mixtures (LS: T₀ = 5.48 *vs* T₆₀ = 5.23; LC: T₀ = 5.80 *vs* T₆₀ = 5.51), very close to the decrease observed (0.30-0.44) in control samples (CLS: T₀ = 5.71 *vs* T₆₀ = 5.27; CLC: T₀ = 5.75 *vs* T₆₀ = 5.45).

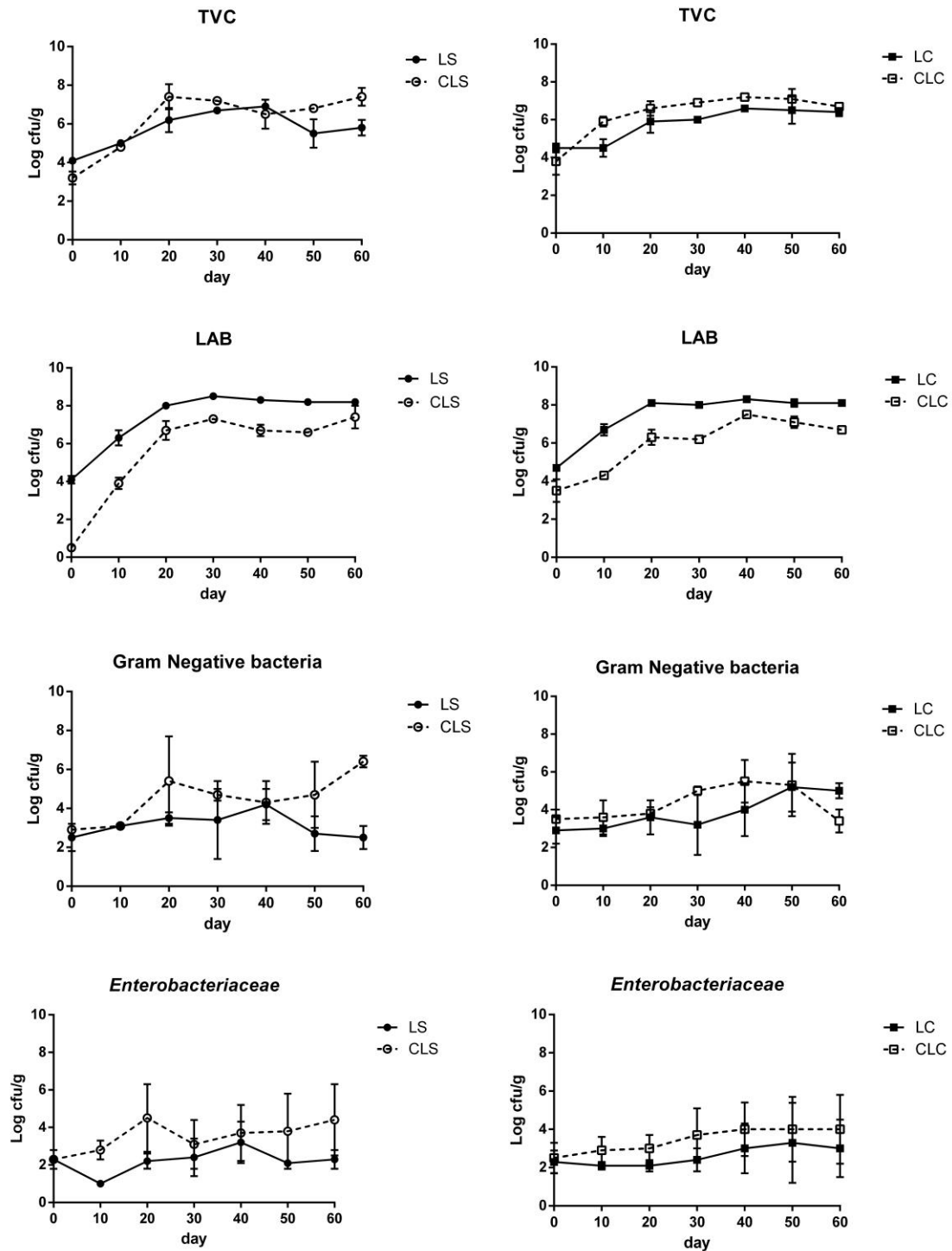


Figure 1 Results of total viable count (TVC), Lactic Acid Bacteria (LAB), Gram negative bacteria and *Enterobacteriaceae*.

CLS= Control samples *L. sakei*; LS= *L. sakei*, inoculated a mixture of the six strains of *L. sakei*; CLC= Control samples *L. curvatus*; LC= *L. curvatus*, inoculated with a mixture of the two strains of *L. curvatus*.

5.4.3 Physical and chemical analyses

Table 2 reports L, a*, b* values and Hue angle detected on the meat surface. Only in few sample times, significant differences were found between LS and CLS and between LC and CLC samples during the experimental period, without any clear trend. The application of LAB cultures did not show to negatively affect meat colour for the whole period considered.

Samples	T0	T10	T20	T30	T40	T50	T60
LS							
L (1h)	44.5±1.7	44.2±1.5	43.1±2.1	42.5±5.5	43.7±2.8 ^B	44.8±2.0	44.8±3.0 ^a
a* (1h)	23.1±2.2	20.8±3.6	20.1±1.2	19.7±1.6 ^B	20.8±1.1 ^A	16.9±2.1	16.9±1.1 ^B
b* (1h)	15.8±1.9	12.8±4.1 ^b	13.3±1.0	13.0±1.0 ^b	14.5±0.3	13.2±0.9	13.4±1.2
Hue-Angle	34.4±1.3	30.7±4.1	33.5±1.4	33.5±2.5	35.0±1.1	38.0±2.2	38.3±0.9
CLS							
L (1h)	44.6±3.5	43.5±1.9	41.7±4.3	44.4±1.9	51.0±1.3 ^A	45.7±1.9	40.9±5.4 ^b
a* (1h)	20.9±1.6	21.7±0.8	19.8±1.1	23.1±3.8 ^A	17.6±3.2 ^B	17.4±1.5	20.0±1.2 ^A
b* (1h)	14.0±1.5	14.9±0.8 ^a	12.4±2.1	15.0±2.2 ^a	14.9±1.2	13.7±1.0	13.7±1.3
Hue-Angle	33.8±0.8	34.4±0.8	31.8±3.4	33.2±3.6	40.7±3.4	38.2±1.4	34.4±2.0
LC							
L (1h)	39.7±3.5	42.0±1.6	44.8±3.8	47.2±3.1	46.3±1.8	42.5±1.4	45.4±6.0
a* (1h)	22.3±2.4	22.8±2.4	20.6±1.4	19.6±1.7	19.0±2.0 ^B	19.3±1.9	21.1±1.8
b* (1h)	13.5±1.1	15.2±2.3 ^A	14.8±1.1	14.5±1.3	13.1±2.2 ^b	14.3±0.6	15.4±1.1
Hue-Angle	31.2±2.3	33.5±2.0	35.7±1.2	36.6±2.3	34.4±2.2	36.7±1.5	36.1±2.3
CLC							
L (1h)	40.7±3.2	42.0±4.9	44.5±1.8	48.2±3.5	43.8±3.1	42.8±3.6	48.2±4.4
a* (1h)	23.0±2.7	20.9±1.3	19.8±1.5	20.2±0.9	22.5±1.6 ^A	21.0±4.0	20.1±1.2
b* (1h)	14.8±2.7	12.4±3.2 ^B	13.4±1.4	14.5±1.2	15.1±1.6 ^a	15.0±1.7	15.2±1.1
Hue-Angle	32.5±2.1	30.2±5.7	34.0±1.1	35.6±1.9	33.7±2.2	35.1±3.8	37.0±1.6

Table 2

Values of L, a*, b* and Hue angle values measured on LS, CLS, LC and CLC samples.

CLS= Control samples *L. sakei*; LS= *L. sakei*, inoculated a mixture of the six strains of *L. sakei*; CLC= Control samples *L. curvatus*; LC= *L. curvatus*, inoculated with a mixture of the two strains of *L. curvatus*.

^{a, b} (P < 0.05); ^{A, B} (P < 0.01)

5.5 Conclusions

Historically *L. sakei* and *L. curvatus* have been recognized for their useful role in food biopreservation by contrasting the growth of spoilage and pathogenic microorganisms without the production of sensorial changes. *L. sakei* mixture and especially *L. curvatus* mixture tested in this work showed promising antimicrobial activity *in vitro* against a wide number of spoilage and pathogenic bacteria. No activity was recorded from the supernatants and the pH adjusted supernatant, for all the target strains tested, highlighting that the antagonistic effect originates probably from the nutrient competitive exclusion and the possible production of metabolites such as organic acids as a combined effect. Moreover, the effect of the addition of the two mixtures to sliced vacuum-packaged beef was investigated, considering microbiological and physical-chemical parameters. The high loads detected on meat even if substantially lower in samples treated with the mixtures if compared with the control ones, could be related to the slighter competitiveness of the cultures if applied to a complex substrate and to the buffering capacity of meat, which decreased the potential action of organic acids. The use of higher dosage of LAB cultures could be suggested as an effective mean to determine an early conditioning of meat environment, in order to prevent the growth of spoilage bacteria and prolong vacuum packaged raw meat shelf-life.

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CHAPTER 6

Fourth study:

Quality and hygiene of beef burgers in relation to the addition of sodium ascorbate, sodium citrate and sodium acetate

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6. Quality and hygiene of beef burgers in relation to the addition of sodium ascorbate, sodium citrate and sodium acetate

AUTHORS: *Simone Stella*¹, *Erica Tirloni*¹, *Barbara Ripamonti*¹, *Francesca Lamanuzzi*², *Patrizia Cattaneo*¹

¹ Department of Health, Animal Science and Food Safety, University of Milan, via Celoria, 10, Milan, Italy

² Fratelli Pagani Spa, via Ennio, 20, Milan, Italy

6.1 Abstract

We evaluated the effects of two additive mixtures (sodium ascorbate 1 g kg⁻¹, sodium citrate 1 g kg⁻¹ and sodium acetate 1.75 or 2.5 g kg⁻¹) on the microbiological and physical-chemical characteristics of non-prepacked beef burgers stored in air at 4°C or 12°C for 96h. Total Microbial Count reached 7 Log CFU g⁻¹ 48h later in treated samples at 4°C. The mixture containing the higher acetate concentration led to a smaller increase in Gram negatives, in particular *Pseudomonas* (2 Log of difference towards control samples at 96h); at 12°C a 1.7 Log difference in *Enterobacteriaceae* was also shown. Total Viable Basic Nitrogen was significantly lower in the treated samples at 12°C. The addition resulted in pH stabilization and lower cooking loss and positively influenced the a* index of burgers at 4°C. Clearly, the use of these mixtures should not be a substitute of good hygienic practices and optimal storage conditions.

6.2 Introduction

One of the major goals of the meat industry is the preservation and consequent extension of a product's shelf life. The application of a combination of mild preservatives ("hurdles"), which do not affect the sensorial characters of raw meat, is considered the most useful method to preserve the microbial safety and stability of meat, as well as its nutritional and sensory quality (Leistner & Gorris, 1995). Several studies have thus evaluated the potential use of organic acids and their salts as meat additives (Östling & Lindgren, 1993; Stivarius *et al.*, 2002; Friedrich *et al.*, 2008). Potential positive benefits were highlighted in terms of

bacterial inhibition and stabilization of sensory characteristics (flavour, colour, juiciness), which are demanded by consumers and required for retail markets.

The antimicrobial action of organic acids and their salts has been widely studied, focusing above all on the inhibition of pathogens such as *Listeria monocytogenes* and *Salmonella* spp. and spoilage microorganisms (*Enterobacteriaceae*, *Pseudomonadaceae*) (Mendonca *et al.*, 1989; Werderquist *et al.*, 1994; Friedrich *et al.*, 2008). Several studies have demonstrated the positive effects of a combination of different organic acids/salts, due to a combination of the specific activities of the different molecules (Drosinos *et al.*, 2006; Jensen *et al.*, 2003a). The addition of organic salts rather than acids has also proven to be interesting for the meat industry as it produces fewer changes to the sensory characteristics of the meat (Mendonca *et al.*, 1989; Ahmed *et al.*, 2003).

The most studied compounds include lactic acid and its salts, in particular sodium and potassium lactates which have an inhibitory action against pathogenic microflora (Seyfert *et al.*, 2007). Recently the antimicrobial effects of other organic compounds such as citric, acetic and ascorbic acid and their salts have been investigated *in vitro* and in meat products (Harris *et al.*, 2012). The antimicrobial activity of acetic acid and its salts is due to their ability to lower the pH and to compromise the bacterial cell walls (Lück & Jager, 1998). Various studies have demonstrated how such organic compounds can counter *Escherichia coli* O157:H7, *Salmonella* Thyphimurium and *Listeria monocytogenes* after being applied to carcasses surfaces, raw meat and meat products (Blom *et al.*, 1997; Conner *et al.*, 1997; Seman *et al.*, 2008). Citric acid and citrates are considered as mild antimicrobial agents, and can be used with other organic acids, resulting in an extension of the lag phase of the autochthonous microorganisms of sheep and goat meat, leading to lower total viable counts (Shelef *et al.*, 1997; Ahmed *et al.*, 2003). A few studies have evaluated the antimicrobial activity of ascorbic acid and its salts, showing that their use as meat additives does not lead to a significant inhibition of spoilage microorganisms (Sahoo & Anjaneyulu, 1997).

In terms of the sensorial quality of meat, colour is the major characteristic influencing purchase decisions at the point-of-sale because customers perceive it as an indicator of freshness. The myoglobin oxidation that takes place while meat is being displayed on shop shelves is in fact responsible for both the brown discolouration and retail price reduction (Saleh & Watts, 1968; Faustman & Cassens, 1990). Acetate is recognized as an excellent shelf life improver, thanks to its marked colour stabilization. Other beneficial effects such as an improvement in tenderness and juiciness of raw meat have also been observed (Mendonca *et al.*, 1989; Jensen *et al.*, 2003a; Seyfert *et al.*, 2007). Citric acid and citrates are commonly used to adjust the pH of food and to avoid myoglobin oxidation (Shelef *et al.*, 1997). Ascorbic acid and ascorbates are added to meat products mainly in terms of their antioxidant activity; in fact they 'regenerate'

meat pigments and maintain sensorial properties for longer periods (Stivarius *et al.*, 2002; Knock *et al.*, 2006; Lund *et al.*, 2007).

EC regulation No. 1129/2011 contains a list of permitted organic acids and salts to be used as additives for meat preparations, following the *quantum satis* principle, which includes ascorbic acid/sodium ascorbate/calcium ascorbate, citric acid/calcium citrate/sodium citrate/potassium citrate, acetic acid/sodium acetate/sodium hydrogen acetate and sodium lactate/potassium lactate. Their addition could be promising for minced meat preparations, which are characterized by high contamination levels after processing and a large surface area that gives rise to high spoilage rates. These preparations are often marketed as prepacked industrial products, often with modified atmosphere packaging (MAP). However a significant share is purchased as non-prepacked products in butcher shops, which are widespread (more than 20,000 shops) throughout Italy, where our study took place. These products are generally prepared at the request of the end consumer, but there is an increasing need for ways to maintain optimal quality and hygiene both while the product is displayed on the shelf and when stored at home. Note that the compounds mentioned above can only be added to prepacked preparations, while no additives are currently permitted for use in non-prepacked minced meat preparations.

The aim of this study was to evaluate the impact of the addition of two mixtures of organic acid salts (sodium ascorbate, sodium citrate and sodium acetate), characterized by a different concentration of sodium acetate, on the shelf life of non-prepacked beef burgers. The burgers were prepared following the typical practice in butcher's shops, and temperature changes during storage were also taken into account.

6.3 Materials and methods

6.3.1 Sample preparation and experimental design

Raw beef cuts (flanks) were purchased from a local retail market and microbiologically analysed to evaluate the initial microbial contamination. NaCl (10 g kg⁻¹) was added to the meat which was minced with a mixer (Novinox, Nova Milanese, I). Three different series of minced meat samples were prepared and added (Table 1). Organic acid salts were provided by Fratelli Pagani Spa (Milan, I).

For each series, twenty-seven burgers each weighing 80 g were formed with a press between two polyethylene sheets. Three samples were analysed immediately, the others were placed in trays covered with a protective film and divided into two groups. The first group was stored at a standard refrigeration temperature (4°C) for 96 h (samples A-B-C). The second group (samples AT-BT-CT) was stored at 12°C: in this way, the possibility of a thermal abuse during

products storage was simulated. Each group of samples was submitted daily (from 0 h to 96 h) to microbiological and physical-chemical analyses in triplicate.

Additive	Temperatures		Sampling time
	4°C	12°C	
Control samples, no additives	A	AT	Microbiological analyses, pH, cooking loss, colour indexes determinations: T0 and after 24, 48, 72 and 96 h. TVBN, a_w: T0 and after 48 and 96 h
Sodium ascorbate (E301) (1 g kg ⁻¹) + sodium citrate (E331) (1 g kg ⁻¹) + sodium acetate (E262) (1.75 g kg ⁻¹)	B	BT	
Sodium ascorbate (E301) (1 g kg ⁻¹) + sodium citrate (E331) (1 g kg ⁻¹) + sodium acetate (E262) (2.50 g kg ⁻¹)	C	CT	

Table 1 Preparation of the samples.

6.3.2 Microbiological analyses

For microbial counts, 10 g of each sample were homogenized in 90 ml of a diluent solution (0.85% NaCl and 0.1% tryptone), and serial 10-fold dilutions were prepared. Total Mesophilic Count (TMC) was determined according to the ISO 4833:2003 method. Gram negative bacteria were enumerated using a spread plate technique on Tryptone Soy Agar (TSA, VWR, Darmstadt, DE) with 10 UI ml⁻¹ of Penicillin G (VWR) added; plates were incubated at 30°C for 48 h. Lactobacilli were enumerated on de Man-Rogosa-Sharpe agar (Oxoid, Basingstoke, UK); plates were incubated at 30°C for 48-72 h under anaerobic conditions (AnaeroGen, Oxoid). The number of *Enterobacteriaceae* was determined by the ISO 21528-2:2004 method. *Escherichia coli* was enumerated according to the ISO 16649-2:2001 method. Coagulase positive Staphylococci were determined by the ISO 6888-1:1999 method. The *Pseudomonas* spp. were enumerated using a spread plate technique on Pseudomonas Selective Agar (PSA, Biolife Italiana, Milan, I); plates were incubated at 30°C for 48 h.

6.3.3 Physical and chemical analyses

The pH of the samples was measured by a pHmeter (mod. XS pH6, Ghiaroni & C., Buccinasco, I). Four repetitions for each sample were performed. The water activity (a_w) was recorded at 0 h, 48 h and 96 h using a Hygrolab (Rotronic Italia, Milan, I). The TVBN (Total Volatile Basic Nitrogen) was determined by the method described in EU Regulation No 2074/2005 at 0 h, 48 h and 96 h.

The surface colour of the meat was assessed daily on six randomly chosen spots of each sample surface using a Minolta CR-200 Chromameter (Minolta, Osaka,

J); L^* (lightness), a^* (“red” index) and b^* (“yellow” index) parameters were determined; Hue angle was also calculated as $\arctan(b^*/a^*) \times 57.29$.

To calculate the cooking loss, samples at 24 h, 48 h, 72 h and 96 h were weighed and cooked to the end-point internal temperature of 70°C on electric countertop griddles preheated to approximately 180°C. The burgers were then kept at room temperature for 15 minutes, dried with absorbent paper, and weighed again.

6.3.4 Statistical analysis

The data from series A, B and C were compared by one-way ANOVA using SAS/STAT package version 8.0 (SAS Inst. Inc., Cary, NC, USA). The same analysis was performed on the data from the series AT, BT and CT. The differences were considered significant at $P < 0.05$ and highly significant at $P < 0.01$.

6.4 Results

6.4.1 Microbiological analyses

The microbiological analyses of the meat cuts used for preparing the burgers revealed the presence of low TMC and lactobacilli counts (4.3 and 4.1 Log CFU g⁻¹, respectively), while *Enterobacteriaceae*, *E. coli* and coagulase-positive staphylococci were < 2 Log CFU g⁻¹.

Figure 1 shows the results of the microbiological analyses of the burgers. Generally, an inhibitory action of the mixtures in terms of bacterial populations was revealed. Considering TMC, it was observed that the level of 7 Log CFU g⁻¹, which is generally considered as a threshold for the initial bacterial deterioration of meat (Jay *et al.*, 1996), was achieved by control (A) samples stored at 4°C after 48 hours, while 96 h were needed to reach the same value for treated (B and C) samples at the same temperature. Thermal abuse (12°C) led, as expected, to a shortened shelf-life. In such conditions, higher counts were found in the control samples during the last part of the trial. Differences of approximately 1 Log CFU g⁻¹ and 1.5 Log CFU g⁻¹ were observed at 72 h and 96 h, respectively, between the controls and treated samples. These differences were statistically significant both at 72 h ($P < 0.05$) and 96 h ($P < 0.01$). Gram negatives represented the major meat bacterial population at the beginning of the storage period, and their rate increased during the study, especially at abuse temperature. The addition of the mixture resulted in a slight inhibition: a significantly lower ($P < 0.01$) load in the CT samples was observed compared to AT (< 1.6 Log) after 96 h of storage at 12°C. The growth of *Enterobacteriaceae* was mainly affected by the storage temperature; at 4°C the concentration of these microorganisms did not show a significant difference throughout the whole trial. At 12°C in the AT samples a

rapid increase in growth was observed starting from 48 h until the end of the experimental trial; a decrease in growth rate was observed in BT and in particular in the CT samples, with significantly lower counts from 48 h to 96 h of storage ($P < 0.01$). Given their psychrotrophic properties, *Pseudomonas* spp. showed a gradual growth in A samples during storage, while in B and C groups, low constant counts were observed for the whole trial; the difference between A and C groups was statistically significant at 48 h of storage ($P < 0.05$) and until the end of the trial ($P < 0.01$ at 72 h and 96 h). At 12°C the inhibitory action of the mixtures against these microorganisms was very evident, with significant differences ($P < 0.01$) already from 24 h of storage; in fact, a difference > 2 Log CFU g⁻¹ was constantly detected between the AT and CT samples. The addition of the mixtures did not inhibit the lactobacilli, which gradually increased during storage in all the samples. Coagulase-positive *Staphylococci* counts did not exceed 2 Log CFU g⁻¹. *Escherichia coli* counts were also below the same level, except at 96 h in samples stored at 12°C, when a difference was observed between the control samples (AT= 4.5 Log CFU g⁻¹) and the treated samples (BT= 3.2 Log CFU g⁻¹ and CT= 2.7 Log CFU g⁻¹).

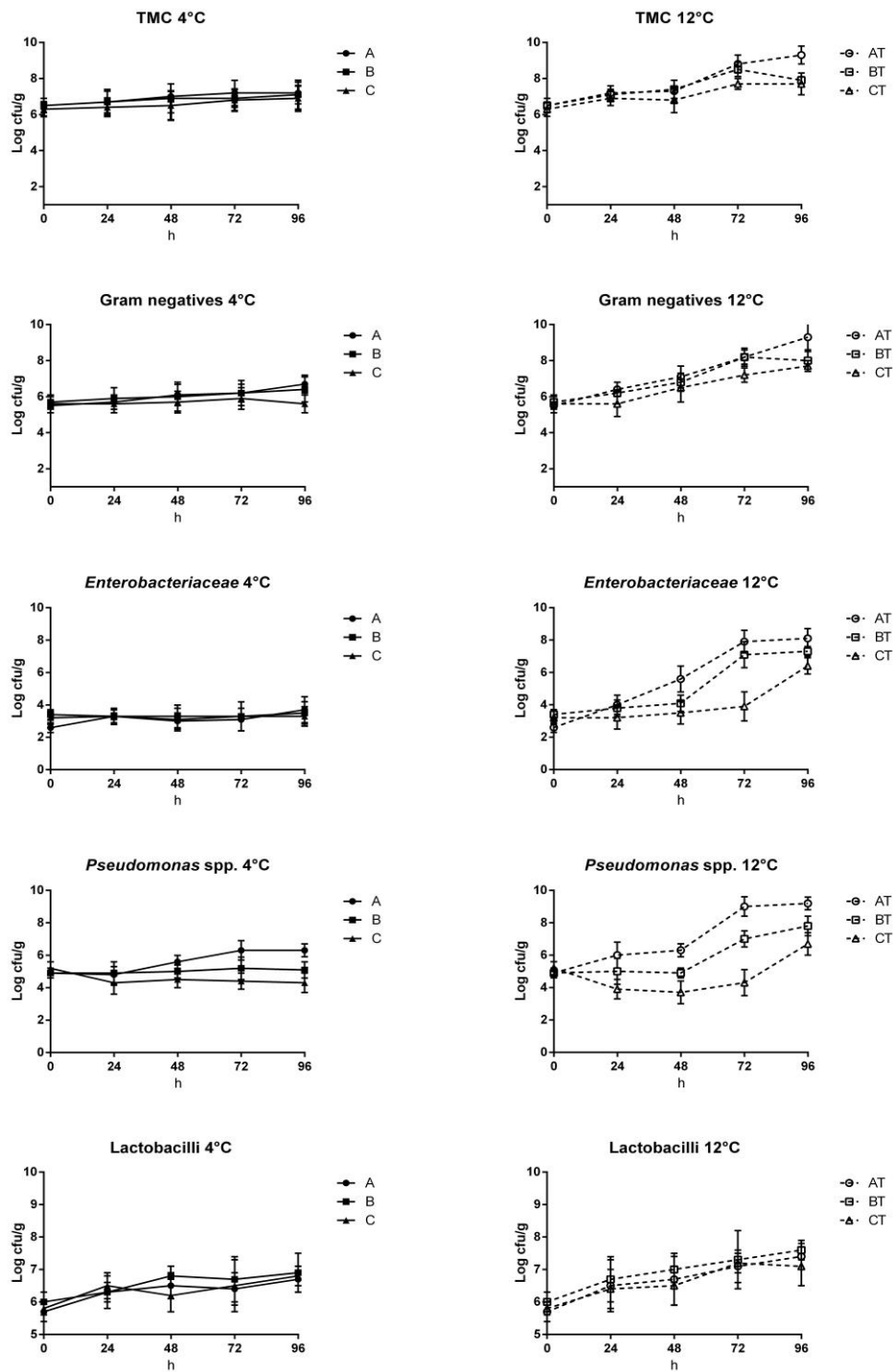


Figure 1: Total Microbial Count, Gram negatives, *Enterobacteriaceae*, *Pseudomonas* spp. and Lactobacilli counts of control (A) and treated samples stored at 4°C (B, C) and control (AT) and treated samples stored at 12°C (BT, CT).

6.4.2 Physical and chemical analyses

In all the samples stored at 4°C (A, B, C), TVBN values increased slightly, without any evident difference between the control and treated groups (Fig. 2). At 12°C, a marked protein degradation was observed in the control samples (AT), while lower TVBN values were detected in the BT and CT samples. These differences became highly significant at 96 h ($P < 0.01$). During the trial, control samples stored at 4°C (A) showed a marked acidification (Fig. 3). This trend has already been described (Mendonca et al., 1989; Drosinos et al., 2006) and was likely due to the activity of the lactobacilli which right from the beginning were present in high counts (about 6 Log CFU/g).

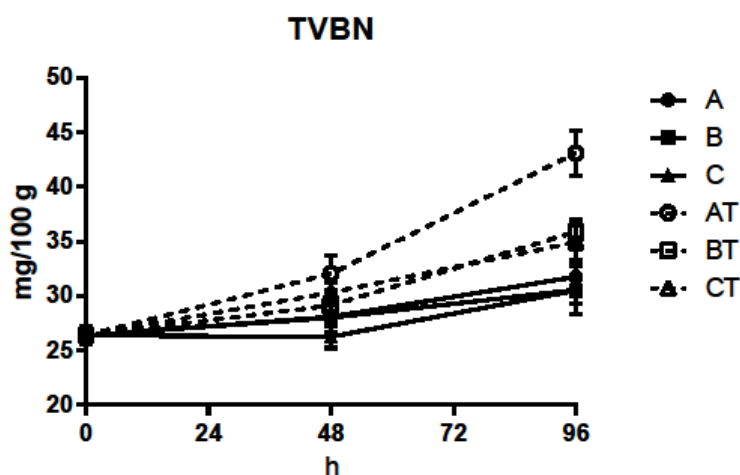


Figure 2: Total Volatile Basic Nitrogen (TVBN) values of burgers stored at 4°C (A, B, C) and 12°C (AT, BT, CT).

The addition of the mixtures resulted in a partial pH stabilization thanks to the presence of organic salts, whose buffering capacity has been already proved during meat storage trials (Jensen et al., 2003a; Mendonca et al., 1989). In AT, BT and CT samples stored at 12°C, an initial acidification was followed by a stabilization of pH values. This trend could be due to the metabolic activity of the microbial population, in particular *Pseudomonas* spp., which catabolize meat lactic acid and produce molecules from muscle protein degradation during storage (Drosinos and Board, 1994).

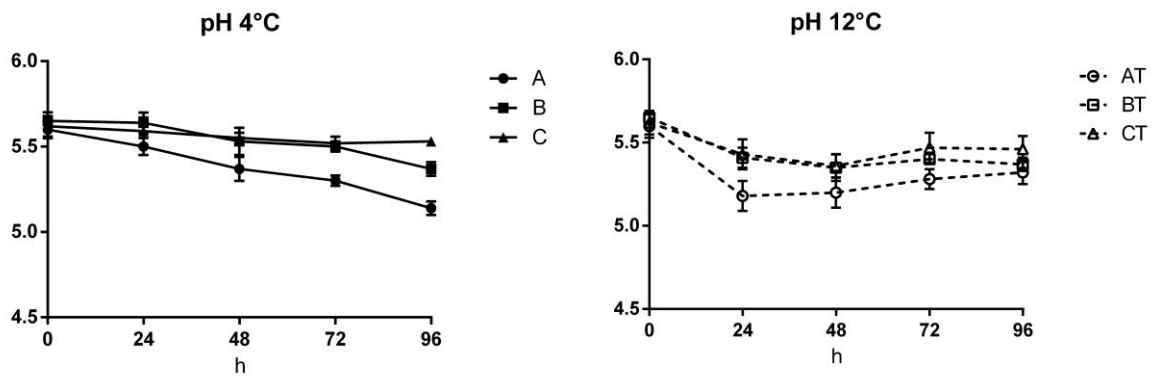


Figure 3: pH of samples stored at 4°C and 12°C.

A_w values were not affected by the addition of the two mixtures, and were stable for the whole trial ranging between 0.98 and 0.99 at both storage temperatures. Table 2 reports a^* and Hue angle values detected on the meat surface. Significantly higher a^* values were detected in treated samples (B, C) compared to the controls (A) immediately after the mixture addition and for the whole storage period at 4°C, highlighting a colour-protective action. In A samples, the red colour decreased gradually and constantly, producing a less desirable greyish and darker colour in the burgers during storage. No significant differences were found between B and C samples during the experimental period. Samples exposed to thermal abuse showed an evident and rapid colour decay: the loss of red colour, associated with the browning of burgers, was observed from 24 h of storage in AT samples, whereas the same phenomenon was revealed after 48 h in BT and CT samples.

The use of the additives positively influenced the cooking loss of the samples. At 4°C treated samples showed slightly higher values at 24 h (A = 18.81%, B = 15.50%, C = 14.37%); at 48 h, this difference increased markedly (A = 24.46%, B = 16.17%, C = 16.32%), with a gradual decrease until the end of the trial (96 h: A = 21.58%, B = 18.28%, C = 17.73%). In thermally abused samples, the difference between the control and treated samples was already high at 24 h (AT = 24.68%, BT = 18.81%, CT = 16.62%), and remained stable throughout the experimental period (96 h: AT = 24.50%, BT = 17.43%, CT = 17.31%).

Parameter	Sample	0 h	24 h	48 h	72 h	96 h
a*	A	11.50 ^{Y,y} ±1.03	9.17 ^Y ±3.15	6.62 ^Y ±2.11	6.19 ^Y ±2.01	4.67 ^Y ±1.24
	B	14.82 ^{X,x} ±1.76	15.21 ^X ±2.21	14.11 ^X ±1.43	12.20 ^X ±2.31	12.50 ^X ±0.76
	C	13.77 ^{x,y} ±1.18	14.76 ^X ±1.98	14.62 ^X ±2.65	13.62 ^X ±1.26	12.28 ^X ±1.14
	AT	-	7.41 ^Y ±1.56	5.18 ^{Y,y} ±1.08	6.80±1.88	8.79±2.34
	BT	-	13.59 ^X ±2.01	9.37 ^X ±1.97	9.10±1.64	9.21±1.12
	CT	-	12.88 ^X ±1.60	7.71 ^x ±0.78	6.89±0.81	8.99±1.87
	Hue angle	A	46.76 ^X ±3.35	50.18 ^X ±1.12	60.96 ^X ±2.90	63.92 ^X ±3.12
B		38.39 ^Y ±3.23	38.52 ^Y ±2.15	38.96 ^Y ±1.57	41.01 ^Y ±2.86	41.05 ^Y ±2.11
C		39.48 ^Y ±2.56	39.02 ^Y ±1.87	39.34 ^Y ±3.19	38.60 ^Y ±3.02	40.95 ^Y ±0.98
AT		-	58.39 ^X ±4.17	66.88 ^X ±1.14	61.06 ^X ±3.78	51.19±3.65
BT		-	37.97 ^Y ±2.68	47.91 ^{Y,y} ±0.67	49.01 ^Y ±2.45	50.48±2.90
CT		-	40.24 ^Y ±3.01	52.85 ^{Y,x} ±1.32	59.68 ^X ±1.78	50.59±2.56

Table 2: Values of a* index and Hue angle measured on control (A) and treated (B, C) samples stored at 4°C and on control (AT) and treated (BT, CT) samples stored at 12°C.

x, y ($P < 0.01$); x, y ($P < 0.05$). Values from different storage temperatures were statistically considered separately.

6.5 Discussion

We evaluated the combined action of mixed organic acid salts on beef burgers prepared in conditions that mimicked how they are generally produced in butcher shops and stored in air. The main factor determining shelf life of beef burgers is the evolution of a microbial population, influenced by initial contamination levels, microbial distribution during preparation, and retail storage/display conditions. Considering the components of our tested mixtures, sodium ascorbate and sodium citrate are not generally associated with considerable antimicrobial effects against spoilage microorganisms, whereas sodium acetate has already shown positive effects in fresh beef, pork, sheep/goat meat, poultry and cooked meat products (Mendonca *et al.*, 1989; Wederquist *et al.*, 1994; Jensen *et al.*, 2003a,b; Dubal *et al.*, 2005, 2007; Drosinos *et al.*, 2006; Seyfert *et al.*, 2007).

The efficacy of sodium acetate was also detected in our study, as an antimicrobial effect proportional to the amount of the sodium acetate concentration was found, particularly towards Gram negative bacteria. A significant inhibitory activity was revealed against both *Enterobacteriaceae* and *Pseudomonas* spp. which represent the most important spoilage microorganisms in raw minced meat stored in aerobic conditions. Unlike in our study, Drosinos *et al.* (2006) also found an antimicrobial action against lactobacilli. The growth of spoilage microorganisms on meat is usually associated with a marked protein degradation, measured by the TVBN index. In our study, this parameter clearly

increased in control samples (AT) stored at 12°C. The action of the additives (probably due to the presence of sodium acetate) countered this trend in treated samples.

The use of additives was also tested for their potential to maintain the optimal sensorial characteristics of fresh ground meat, thus extending its “sensorial shelf-life”. Our focus was on the red colour of the burgers, which consumers perceive as an indicator of freshness, especially in retail sales where non-prepacked preparations are usually purchased. The addition of sodium ascorbate, sodium citrate and sodium acetate positively influenced the permanence of the redness. This effect was due to the concurrent action of antioxidants (ascorbate/citrate) and sodium acetate; the latter being known for its significant effect on protecting the colour, although how this actually takes place is not well known.

The samples with higher microbial counts, detected in the last days of the study, were characterized by evident discolouration (browning); consequently, those products were not suitable for sale. These results suggest that the protection of the colour cannot mask any microbial deterioration. This is certainly positive as it acts as a deterrent to fraudulent sales.

Our results also highlighted a buffering effect and a decrease in cooking loss of the treated samples, with a positive influence on the juiciness of the meat perceived by the consumer (Jensen *et al.*, 2003a; Knock *et al.*, 2006; Ke *et al.*, 2009). In any case, the addition of sodium ascorbate, sodium citrate and sodium acetate did not significantly modify the physico-chemical properties of these products, as the typical perishability of fresh meat preparations was maintained.

6.6 Conclusions

High consumer demand for beef burgers requires measures to minimize microbial spoilage during manufacturing, as well as effective means to ensure sensorial stability during their shelf life. Our data indicated that the addition of mixtures containing sodium ascorbate, sodium citrate and sodium acetate was effective in microbial inhibition, especially when the acetate dose was 2.50 g kg⁻¹. Its activity was particularly evident on Gram negative bacteria which are known to be more susceptible to organic acids and represent the most important microorganisms in determining the shelf life of raw minced meat. This activity, together with a significantly slower protein degradation rate, resulted in an improvement in the hygienic quality of treated meat.

Despite the positive results on the maintenance of sensorial characteristics, the addition did not mask any microbiological deterioration of the minced meat, thus minimizing the risk of consumer deceit. It could therefore be very useful for butchers to extend the use of these organic salts also to non-prepacked

minced meat preparations (burgers, meat patties, etc.), as an additional means to ensure product safety and shelf-life.

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CHAPTER 7

General Discussion

7. General Discussion

Different alternative or complementary preservation technologies have been studied and developed in the last years for food conservation. Many approaches have been considered and consequently many promising technologies were currently evaluated in industrial production.

In these studies, different approaches for the conservation of meat and fish products were evaluated, with particular attention to the application of bioprotective cultures and the addition of weak organic salts, following the demands of the consumers for the use of mild technologies in food processing, able to contrast the pathogenic and spoilage microorganisms but at the same time guaranteeing the natural appearance of the product.

As already mentioned, the application of bioprotective cultures to enhance the microbial quality of foodstuff is one of the most promising tools: during these studies, different strains were considered for bioprotective capabilities. We decided to deepen the abilities and behaviour of autochthonous microorganisms, present on several substrates.

From cold smoked salmon scraps, autochthonous LAB were isolated and tested for antilisterial activity; the same approach was followed for vacuum packaged Argentine meat, testing *in vitro* and on meats indigenous LAB isolated from long-stored beef. Furthermore, microorganisms from non-food origin (isolated from the gut of veal calves) already evaluated for probiotic attitudes, were tested newly for antimicrobial activity *in vitro* for a further use in vacuum-packed meat.

An *in vitro* anti-listerial activity of some of *Lactobacillus sakei* strains, isolated from salmon scraps, was detected. Furthermore, *Lactobacillus animalis* SB310 and *L. paracasei* subsp. *paracasei* SB137, never studied before for bioprotective purpose and characterized by limited knowledge regarding their antagonistic properties, showed promising antimicrobial activity *in vitro* towards several Gram positive and Gram negative bacterial species (*Escherichia coli*, *Escherichia coli* 0157:H7, *Proteus vulgaris*, *Salmonella* Typhimurium, *Serratia marcescens*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Listeria monocytogenes*, *Listeria innocua* and *Staphylococcus aureus*). In addition, *L. sakei* mixture and especially *L. curvatus* mixture showed a promising antimicrobial activity against the same spoilage and pathogenic bacteria, showing some matching results in terms of susceptibility of the target strains (i.e *Pseudomonas* spp. resulted the most susceptible and *S. marcescens* the most resistant strains).

The antagonistic activity of LAB is mainly reported to be due to the competition for nutrients and to the production of antimicrobial compounds such as bacteriocins, reuterin, organic acids (mainly acetic and lactic acids), carbon dioxide, diacetyl, ethanol, hydrogen peroxide and enzymes.

The mechanism of action of all the strains tested in our works resulted to be fairly equivalent and it was based on the competitive exclusion, as the presence of live cells was needed to exert an evident microbial action. Moreover, these strains evidenced an acidifying activity that could be related to the production of organic acids; these acids alone, in any case, were not able to produce an antagonistic activity as evidenced by the *in vitro* results where the absence or very limited wideness of the inhibition halos produced by the cell-free supernatants was detected.

Several authors underlined that LAB strains often suffer from a limited efficiency in foods, principally due to the poor adaptation to food environment, to the buffering capacity of meat and to the inactivation of antimicrobial compounds produced by bioprotective cultures. Considering the addition of bioprotective cultures to meat, *L. sakei* mixture (of six strains) and *L. curvatus* mixture (of two strains) determined an inhibitory effect on TVC, *Enterobacteriaceae* and Gram negative bacteria even if the loads remained high, confirming a slighter effect if compared with *in vitro* results.

On the other hand, several studies have evaluated the potential use of mild organic acids and their salts as meat additives, evidencing potential positive benefits in terms of bacterial inhibition and stabilization of sensory qualities, which are demanded by consumers and required for retail markets. The addition to beef hamburgers of mixtures containing sodium ascorbate, sodium citrate and sodium acetate were effective determining a microbial inhibition, especially when the acetate dose was higher. This, associated with lower TVB-N values, resulted in an improvement in the hygienic quality of treated meat. These salts are now admitted, following the Reg. EC n. 1333/2008 and Reg. EU 1129/2011, only for “prepacked meat preparations” but the results evidenced the opportunity to enlarge their use to non-prepacked minced meat preparations as an additional means to ensure product safety and shelf-life.

The evident results obtained with bioprotective culture and organic acids, applied to improve safety and quality of food products, clearly showed that the application of these mild technologies suggest several important advantages like: the enhancement of shelf life, the decreased risk of transmission of pathogenic microorganisms, the reduction of the economic losses due to food spoilage and a good answer to consumer demands for minimally processed foods.

CHAPTER 8

Appendix

8 Other scientific production

8.1 Screening of species-specific lactic acid bacteria for veal calves multi-strain probiotic adjuncts

By Ripamonti Barbara, Agazzi Alessandro, Bersani Carla, De Dea Paola, Pecorini Chiara, Pirani Silvia, Rebucci Raffaella, Savoini Giovanni, Stella Simone, Stenico Alberta, Tirloni Erica, Domeneghini Cinzia.

Published in Anaerobe, 2011, 17, 97–105.

Abstract: The selection of promising specific species of lactic acid bacteria with potential probiotic characteristics is of particular interest in producing multi species-specific probiotic adjuncts in veal calves rearing. The aim of the present work was to select and evaluate *in vitro* the functional activity of lactic acid bacteria, *Bifidobacterium longum* and *Bacillus coagulans* strains isolated from veal calves in order to assess their potential use as multi species-specific probiotics for veal calves. For this purpose, bacterial strains isolated from faeces collected from 40 healthy 50-day-calves, were identified by RiboPrinter and 16s rRNA gene sequence.

The most frequent strains belonged to the species *B. longum*, *Streptococcus bovis*, *Lactobacillus animalis* and *Streptococcus macedonicus*. Among these, 7 strains were chosen for testing their probiotic characteristics *in vitro*. Three strains, namely *L. animalis* SB310, *Lactobacillus paracasei* subsp. *paracasei* SB137 and *B. coagulans* SB117 showed varying individual but promising capabilities to survive in the gastrointestinal tract, to adhere, to produce antimicrobial compounds.

These three selected species-specific bacteria demonstrated *in vitro*, both singularly and mixed, the functional properties needed for their use as potential probiotics in veal calves.

8.2 Effects of a species-specific probiotic formulation on multiresistant *Escherichia coli* isolates from the gut of veal calves

by Ripamonti Barbara, Tirloni Erica, Stella Simone, Bersani Carla, Agazzi Alessandro, Marocco Serena, Savoini Giovanni.

Published in Czech Journal of Animal Science, 2013, 58, 201–207.

Abstract: In this study, 254 *Escherichia coli* isolates from faecal samples of veal calves were evaluated for antimicrobial susceptibility using the disk diffusion method. During the experimental period, six mass antibiotic treatments were administered to the animals (about one treatment per month). The active principles used were oxytetracycline, colistin, tylosin, doxycycline, chlortetracycline, and sulphonamides. An extremely high resistance prevalence (>70%) towards penicillin, sulphonamide, tetracycline, ampicillin, and spyramicin was detected. Sixty *E. coli* isolates could be defined as multiresistant, showing resistance to at least 6 antimicrobial classes. Subsequently, we evaluated the inhibitory effect of a species-specific probiotic against multiresistant *E. coli*, showing its beneficial action with large inhibition halos for 76% of the isolates. This suggests the potentiality of the probiotic, putting in evidence a clear advantage of its use in veal calves nutrition, in particular during the first phases, when the animals are more susceptible to severe enteric infections by *E. coli*.

8.3 Effect of dietary species-specific probiotic in newborn female calves during the first month of life on intestinal Lactobacilli and *Escherichia coli* populations, health status, and cell mediated immune response

by Agazzi Alessandro, Tirloni Erica, Stella Simone, Maroccolo Serena, Ripamonti Barbara, Bersani Carla, Caputo Jessica Michela, Dell'Orto Vittorio, Rota Nicola, Savoini Giovanni.

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Abstract: The aim of this study was to evaluate the effects of the administration of a species-specific probiotic (*Lactobacillus animalis* SB310-*Lactobacillus paracasei* subsp. *paracasei* SB137-*Bacillus coagulans* SB117 in a 30:35:35 ratio, respectively; 1.8×10^{10} CFU/g of powder) on gut microbial balance, immune response and growing performance of Holstein female calves for the first month of life. Twenty-two calves were divided in two experimental groups from the second until day 28 of life: control (C), fed with milk replacer and concentrate as a basal diet, and treatment (T), fed C diet plus 1g/calf/d of probiotic powder for the first month of age. Fecal and blood samples were individually collected and analysed weekly. Individual fecal score was recorded daily and general health score was calculated at the end of the trial. Cell mediated immune response was evaluated by skin test at 7 and 28 days of life. Milk replacer and concentrate intake were recorded daily, while body weight and biometrical parameters were recorded at 2, 8, 14, 21 and 28 days of life, thus average daily gain and feed conversion rate were calculated. During the first week of treatment, lower blood eosinophils percentage (0.05% *vs.* 0.22%; $P \leq 0.01$) was found in T group, while basophils were higher in T than C at the end of the trial (0.21% *vs.* 0.16%; $P \leq 0.05$). Higher fecal Lactic Acid Bacteria (LAB)/*E. coli* ratio on day 28 of life (3.73 Log CFU/g *vs.* 2.02 Log CFU/g; $P \leq 0.05$) and lower incidence of diarrhoea were found in treated group (63.30% *vs.* 70.71%; $P = 0.05$). Body weight (48.92 kg *vs.* 46.92 Kg; $P \leq 0.05$), total concentrate intake (14.77 Kg *vs.* 12.56 Kg on dry matter basis; $P \leq 0.05$), and hearth girth (81.16 cm *vs.* 78.49 cm; $P \leq 0.05$) were significantly higher in T group. The administration of the probiotic during the first month of life improved gut microbiota and increased the growth performance and some biometric parameters of calves.

CHAPTER 9

Summary

9. Summary

In these studies, different approaches for the conservation of meat and fish products were deepened. The application of bioprotective cultures and the addition of organic salts were investigated, following the current trends of demanding mild technologies for food, in order to contrast the pathogenic and spoilage microorganisms without significant modifications of the sensorial quality of the products.

In the first study, the anti-listerial activity of *L. sakei* strains, isolated from salmon scraps, was observed evidencing the importance of the possibility to reduce the risk of food alteration in cold smoked salmon industry and to prevent the replication of *L. monocytogenes* using autochthonous microorganisms with a potential biopreservative action.

Moreover, in the second study, strains of *Lactobacillus animalis* and *L. paracasei* subsp. *paracasei*, never studied before for bioprotective purpose and characterized by limited knowledge regarding their antagonistic properties, showed promising antimicrobial activity *in vitro*. This action against a wide number of spoilage and pathogenic bacteria was detected both when the strains were used singularly and was more evident when they were applied as mixture (especially when *L. paracasei* subsp. *paracasei* was predominant). The antagonistic effect was due mainly to the competition for nutrients and to the production of organic acids (acetic and lactic) as combined effects.

In the third study, two multistrain mixtures (*L. sakei* mixture and especially *L. curvatus* mixture), obtained adding different strains isolated and identified from Argentine vacuum packed meat, showed a promising antimicrobial activity against a wide number of spoilage and pathogenic bacteria, highlighting that the antagonistic effect originates probably from the nutrient competitive exclusion and the possible production of metabolites such organic acids. Moreover, the addition of the two mixtures to sliced vacuum-packed beef determined a better microbial quality of meat as TVC, *Enterobacteriaceae* and Gram negative bacteria resulted lower if compared to control samples. In any case, if compared with *in vitro* results, a slighter effect was detected on beef samples; it was probably due to the different competitiveness of the cultures if applied to a complex substrate like meat and to the buffering capacity of meat.

The fourth study was focused on the application of organic acids and salts on non-prepacked hamburgers, as they are known to exert potential benefits in terms of bacterial inhibition and stabilization of sensory characteristics. The addition of mixtures containing sodium ascorbate, sodium citrate and sodium acetate was effective in microbial inhibition of non-prepacked beef hamburgers, especially when the highest acetate dose was used. An evident activity was found against Gram negative bacteria, which represent the most important microorganisms in determining the shelf-life of raw minced meat. This,

associated with lower TVB-N values, resulted in an improvement in the hygienic quality of treated samples. These salts are now admitted only for “prepacked meat preparations” but the results evidenced the convenience for butchers to extend the use of these organic salts to non-prepacked minced meat preparations as an additional means to ensure product safety and shelf-life.

In conclusion, the two technologies tested in these studies resulted to be promising as an evident antimicrobial effect was confirmed, suggesting the possibility to apply these tools to extend the shelf-life of food.

CHAPTER 10

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