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Contents

Chapter 1
Introduction

Microbal foodborne diseases are a constant concern to human health, as shown by annual statistics published by official institutions (http://www.cdc.gov/foodborne-burden/; http://www.efsa.europa.eu/en/efsajournal/pub/3129.htm). The globalization of the food market and the large-scale distribution and processing of raw materials and food products create new ecological niches to which microorganisms from different regions of the world may adapt, raising new problems that the food industry must solve. This trend increases as the food chain tends to be more complex in several ways, including transportation distance, processing steps, distribution of raw materials, and shelf life extension of the finished products. The increase of the more susceptible populations (e.g., the young, elderly, and immunocompromised individuals), the migration of populations from rural to urban areas, the overexploitation of natural resources (such as soil and water) and the climatic changes, are also factors to be taken into consideration. The food industry also has to satisfy the newer consumer habits. In the past years, there has been a growing demand of consumers for foods that are fresh-tasting, lightly preserved, ready-to-eat, and (possibly) with health-promoting effects. Consumer organizations are also more and more concerned about the quality of foods and the ways in which they are produced.

Although some traditional methods for food production and processing are being abandoned, there is also a growing interest in traditional foods, and in the adaptation of local production processes to an industrial scale without substantial loss of the original value. Several developing countries already benefit from modern food industry; however, in many others, there is an overwhelming need to enhance the availability of raw materials and to promote food processing on an industrial scale in order to meet the nutritional requirements of the population and to provide a minimal framework of food safety. The need to avoid economic losses due to microbial spoilage of raw materials and food products, to decrease the incidence of foodborne illnesses, and to meet the food requirements of the growing world population strengthen the relevance of preservation methods in the food industry. In this context, the preservation of foods by natural, biological methods may be a satisfactory

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approach to solve many of the current food-related issues. All these factors have stimulated scientific research to exploit natural weapons, either alone or in combination with novel food processing technologies, in the development of biopreservation strategies compatible with the latest changes in human habits and lifestyle.

Chapter 2
Natural Antimicrobials for Food Biopreservation

Biopreservation or biocontrol refers to the use of natural or controlled microbiota, or its antibacterial products to extend the shelf life and enhance the safety of foods (Stiles 1996). Since lactic acid bacteria (LAB) occur naturally in many food systems and have a long history of safe use in fermented foods, thus classed as Generally Regarded As Safe (GRAS), they have a great potential for extended use in biopreservation. Antimicrobial substances from other natural sources, such as antimicrobial proteins or peptides from animal secretions, or bioactive molecules from plant or animal defense systems have also been exploited in different ways for food biopreservation.

2.1 Bacterial Antagonism as a Fundamental for Biopreservation

Microbes often live in complex ecosystems where they must interact with the biotic and abiotic components of the environment. Bacterial populations must compete for space and nutrients in order to survive. They have evolved different mechanisms such as nutrient and space competition, metabolic specialization, o cell differentiation, among others. One of the most common strategies to defend a population territory is ammensalism, which is based on the modification of the environment by the release of antimicrobial substances that inhibit growth or even kill competitors. Bacteria may release a variety of antimicrobial substances as byproducts of their normal metabolic activity. They also may produce more specific, dedicated antimicrobial weapons encoded by specific genetic determinants aimed specifically at combating other microbes. Metabolic products as well as antimicrobial peptides from lactic acid bacteria (LAB) have attracted great attention for food biopreservation. Being naturally or intentionally present in food fermentations, the lactic acid bacteria are considered themselves as natural preservatives as well as factories of

A. Gálvez et al., *Food Biopreservation*, SpringerBriefs in Food, Health, and Nutrition, DOI 10.1007/978-1-4939-2029-7_2

natural antimicrobials for food biopreservation. LAB may produce a wide variety of active antagonistic metabolites such as organic acids (lactic, acetic, formic, propionic, butyric, hydroxyl-phenyllactic acid, and phenyllactic acid), diverse antagonistic compounds (carbon dioxide, ethanol, hydrogen peroxide, fatty acids, acetoin, diacetyl, reuterin, reutericyclin), antifungal compounds (propionate, phenyl-lactate, hydroxyphenyl-lactate, cyclic dipeptides, phenyllactic acid and 3-hydroxy fatty acids), and bacteriocins (such as nisin, pediocins, lacticins, enterocins and many others) (Muhialdin et al. 2011; Reis et al. 2012; Oliveira et al. 2014). Other bacterial groups (especially those from genus *Bacillus*) are also attracting attention because of the diversity of antimicrobial peptides they produce, some of which could also be exploited as biopreservatives.

2.1.1 *Antimicrobial Substances Derived from Bacterial Cell Metabolism*

Organic acids. Fermentation is an oxido-reductive process in which organic acids are the main end products. Lactic acid is the main organic acid produced during fermentation. Organic acids decrease the pH of the surrounding environment, creating a selective barrier against non-acidophiles. In addition, organic acids also have antibacterial activity. The antimicrobial effect of lactic acid is exerted by disruption of the cytoplasmic membrane and interference with membrane potential (Axe and Bailey 1995) and/or reduction in intracellular pH (Shelef 1994).

CO_2. Heterofermentative LAB produce CO_2 as a byproduct of sugar fermentation. The production of CO_2 creates an anaerobic environment has antagonistic effects specifically against aerobic bacteria (Adams and Nicolaides 1997). It also dissolves in water, generating carbonic acid.

Diacetyl. Certain LAB may produce diacetyl (2,3-butanedione) is a by-product of the metabolic activity (Jay 1982). Diacetyl exhibits antibacterial activity against *Listeria, Salmonella, Escherichia coli, Yersinia,* and *Aeromonas* (Jay 1982). Gram-negative bacteria are generally more sensitive than gram-positive bacteria to diacetyl (Adams and Nicolaides 1997). However, since the high concentrations of diacetyl required to achieve inhibition of spoilage bacteria also affect the sensory properties of the food (Helander et al. 1997), the use of diacetyl-producing cultures for protective purposes should be limited to foods where diacetyl is an essential component of the food sensory properties (Jay 1982).

Hydrogen peroxide. LAB are deficient in catalase activity. Hydrogen peroxide is produced in the presence of oxygen as a result of the action of flavoprotein oxidases or NADH peroxidase (Ammor et al. 2006). Hydrogen peroxide is thought to elicit an antibacterial effect through oxidative damage of proteins, but it also may increase membrane permeability (Kong and Davison 1980).

Reuterin. Also known as 3-hydroxypropionaldehyde (3-HPA), reuterin is a low-molecular-weight antimicrobial compound produced by *Lactobacillus reuteri*

(Talarico et al. 1988) and some other LAB. It is formed as an intermediate during the metabolism of glycerol to 1,3-propanediol under anaerobic conditions (Talarico et al. 1988; Talarico and Dobrogosz 1989) and behaves in solution as an equilibrium mixture of monomeric, hydrated monomeric and cyclic dimeric forms of 3-HPA. The antimicrobial activity of reuterin has been attributed to its ability to inhibit DNA synthesis (Talarico and Dobrogosz 1989), being active on bacteria as well as yeasts and molds. Reuterin has broad spectrum of activity and inhibits fungi, protozoa and a wide range of bacteria including both gram-positive and gram-negative bacteria. It has bacteriostatic activity against *L. monocytogenes* and variable bactericidal activities against *Staphylococcus aureus*, *E. coli* O157:H7, *Salmonella* Choleraesuis, *Yersinia enterocolitica*, *Aeromonas hydrophila* subsp. *hydrophila,* and *Campylobacter jejuni* (Arqués et al. 2004).

Reutericyclin. This unique tetramic acid is a negatively charged, highly hydrophobic antagonist (Gänzle et al. 2000). Reutericyclin acts as a proton ionophore, resulting in dissipation of the proton motive force (Gänzle 2004). It lacks activity towards yeasts and fungi, but it is active on gram-positive bacteria including *Lactobacillus* spp., *Bacillus subtilis, Bacillus cereus, Enterococcus faecalis, S. aureus* and *Listeria innocua.* Spore germination of *Bacillus* species was inhibited by this antimicrobial compound, but the spores remained unaffected under conditions that do not permit germination. As in many other antagonists, inhibition of Gram-negative bacteria (*E. coli* and *Salmonella*) is observed under conditions that disrupt the outer membrane, including truncated lipopolysaccharides (LPS), low pH and high salt concentrations. Reutericyclin was shown to be produced in concentrations active against competitors during growth of *Lactobacillus reuteri* in sourdough. It was proposed that reutericyclin-producing strains may have applications in the biopreservation of foods (Gänzle 2004).

2.1.2 Antifungal Compounds

The only antifungal compound approved for food applications is derived from an actinomycete. Natamycin (pimaricin) is an antifungal compound produced by *Streptomyces natalensis,* approved as a broad-spectrum antifungal biopreservative for foods and beverages (Stark 2003). Natamycin binds irreversibly to the cell membrane of fungi because of its high affinity for ergosterol. This causes membrane hyperpermeability leading to rapid leakage of essential ions and peptides and ultimately cell lysis (Teerlink et al. 1980). There is a growing interest in antifungal compounds from LAB, and a few LAB strains showing antifungal activities have been characterized regarding the antimicrobial substances responsible for the observed inhibitions. Usually, antifungal activity produced by LAB is due to the combination of organic acids and cyclic dipeptides. Besides lactic acid, other organic molecules such as phenyllactic and 4-hydroxy-phenyllactic acids (Dal Bello et al. 2007; Lavermicocca et al. (2000, 2003)), benzoic acid, methylhydantoin, mevalonolactone (Niku-Paavola et al. 1999), 5-oxododecanoic acid, 3-hydroxy

decanoic acid and 3-hydroxy-5-dodecenoic acid (Ryu et al. 2014) or 3,6-bis(2-methylpropyl)-2,5-piperazinedion (Yang and Chang 2010) have been identified. Among the cyclic dipeptides described in cell-free supernatants of antifungal LAB strains are cyclo (Gly-LLeu) (Niku-Paavola et al. 1999), cyclo (L-Leu-L-Pro) and cyclo (L-Phe-L-Pro) (Dal Bello et al. 2007), cyclo (L-Phe-l-Pro) and cyclo (L-Phe-trans-4-OH-L-Pro) (Ström et al. 2002). Some of them require high concentrations in order to be effective, while others have demonstrated potential for biopreservation in certain foods such as bread (Dal Bello et al. 2007).

2.1.3 Bacteriocins

Bacteriocins can be defined as ribosomally synthesized antimicrobial peptides or proteins, which can be posttranslationally modified or not (Jack et al 1995). Bacteriocins from Gram-positive bacteria are generally classified according to size, structure, and modifications. Klaenhammer (1993) defined four classes of bacteriocins produced by LAB. Class I bacteriocins or 'lantibiotics' are small, ribosomally synthesized peptides that undergo extensive post-translational modification. They contain lanthionine and b-methyl lanthionine residues, as well as dehydrated amino acids. Class II bacteriocins are small (4–6 kDa), heat-stable, ribosomally synthesized peptides which were differentiated from lantibiotics because they do not undergo extensive post-translational modification, except for cleavage of a leader peptide (when present) during transport out of the cell. Nevertheless, some exceptions to this rule have been reported recently, illustrated by the n-terminal formylated two-peptide bacteriocin from *E. faecalis* 710C (Liu et al. 2011) and enterocin BacFL31 which contains hydroxyproline residues (Chakchouk-Mtibaa et al. 2014). Nes et al. (1996) regrouped the class II bacteriocins, retaining class IIa and IIb but changing class IIc to include bacteriocins that contain a typical signal peptide and that are secreted by the general translocase (*sec*) pathway of the cell. Cotter et al. (2005) suggested to divide class II bacteriocins into several subclasses: class IIa (pediocin-like bacteriocins), class IIb (two-peptide bacteriocins), and class IIc (circular bacteriocins) Cotter et al. (2005). However, circular bacteriocins may also be considered as a separate class (Franz et al. 2007; van Belkum et al. 2011). Nonbacteriocin lytic proteins, termed bacteriolysins (also referred to as class III bacteriocins), are large and heat-labile proteins with a distinct mechanism of action from other Gram-positive bacteriocins (Cotter et al. 2005). Specific classification schemes were also proposed for bacteriocins from genus *Enterococcus* (Franz et al. 2007) and genus *Bacillus* (Abriouel et al. 2011). This last one generates a wide variety of peptide structures containing modified amino acid residues other than the classical ones found in, for example, nisin. An updated classification of bacteriocins was proposed by Rea et al. (2011) including two additional subclasses for the lantibiotics and one additional subclass for the non-modified peptides. An orientative summary on the diversity of bacteriocins from Gram-positive bacteria is presented in Table 2.1.

Table 2.1 Classes of bacteriocins produced by Gram-positive bacteria

Class	Features	Type or subclass	Examples
Class I (lantibiotics)	Posttranslational modification yielding unusual amino acid residues	Type A: Cationic, amphiphilic, pore forming activity on the bacterial membrane	Nisin A/Z, lacticin 481, lacticin 3147, subtilin, plantaricin C, varicin 8, lactocin S
		Type B: Globular, no or negative charge, inhibit phospholipase A2	Mersacidin, duramycin B/C, cinnamycin
		Labyrinthopeptides: contain labionin, a carbocyclic, post-translationally modified amino acid residue	Labyrinthopeptins A1, A2, and derivatives
		Sactibiotics: Contain intramolecular sulfur to a-carbon crosslinkages	Subtilosin A, thuricin CD
Class II (nonlantibiotic peptides)	No posttranslational modification, small cationic, amphiphilic peptides[a]	Class IIa: Antilisterial, pediocin-like bacteriocins (YGNGV motif)	Pediocin AcH/PA-1, sakacin A, curvacin A, enterocin A
		Class IIb: Two-peptide bacteriocins[b]	Enterocin L50, lacticin F, lactococcin G and Q; plantaricins EF and JK
		Class IIc: Circular bacteriocins	Enterocin AS-48, reutericin 6, gassericin A, lactocyclin Q
		Class IId: Other single-peptide, nonpediocin molecules[b]	Lactococcins A and 972, enterocin EJ97, divergicin A
Class III (bacteriolysins)	Nonbacteriocin lytic proteins, large, heat labile, cause cell lysis through cell wall hydrolysis		Lysostaphin, helveticin J, enterolysin A

[a]Some may contain modified amino acid residues like n-terminal formylation or hydroxyproline
[b]Some of these are synthesized without leader peptide, and could be included in a separate class of leaderless bacteriocins

2.2 Antimicrobials from Animal Sources

Antimicrobial proteins and peptides are naturally found as part of the defense system of living organisms (including humans, animals, plants, insects…). Lysozyme, lactoferrin and ovotransferrin are illustrative examples. Lysozyme from different sources is commercialized as a natural preservative for food applications, either singly or in combination with other antimicrobials. Lysozyme is generally recognized as safe (GRAS) for direct addition to foods (FDA 1998). Lactoferrin (and its partial hydrolysis derivative lactoferricin) is another natural protein (which is found in milk and other secretions) with antimicrobial activity due to its iron-binding capacity and polycationic nature (Ellison 1994). Lactoferrin shows antimicrobial activity against a wide range of bacteria (including foodbone pathogens like *Carnobacterium, L. monocytogenes, E. coli*, and *Klebsiella*) and viruses (Lönnerdal 2011; Gyawali and Ibrahim 2014), and has been approved for application on beef in the United States and has been applied as an antimicrobial in a variety of meat products (Juneja et al. 2012; USDA-FSIS 2010).

Lactoperoxidase is another antimicrobial system that originated from milk and is reported to be effective against gram-negative bacteria (de Wir and van Hooydonk 1996). Ovotransferrin has a high affinity for iron, and inhibits bacterial growth due to iron deprivation (Valenti et al. 1987). Interestingly, hydrolysis of natural proteins may yield peptide fragments with diverse biological activities, including antimicrobial activity (Möller et al. 2008). Following a strategy of "tailoring and modelling," a number of short peptides with high bactericidal activity have been developed from the bactericidal domain of lysozyme. Ovotransferrin, alpha-lactalbumin and beta-lactoglobulin have also been investigated as sources of antimicrobial peptides (Pellegrini 2003).

Protamine is composed of cationic antimicrobial peptides naturally present in spermatic cells of fish, birds and mammals (Rodman et al. 1984) and is commercially recovered from herring (clupeine) and salmon (salmine) milt. With a MW of 4,112 Da and a pI of 11–13, protamine is the most cationic naturally occurring cationic antimicrobial peptide described to date (Potter et al. 2005). It shows broad antimicrobial activity against gram-positive bacteria, gram-negative bacteria, and fungi (Uyttendaele and Debevere 1994). Protamine has been used to preserve a wide variety of foods ranging from confection items to fruits and rice.

Pleurocidin is present in myeloid cells and mucosal tissues of many vertebrates and Invertebrates (Jia et al. 2000). It shows antimicrobial activity against several foodborne bacteria, such as *L. monocytogenes* and *E. coli* O157:H7, and pathogenic fungi (Burrowes et al. 2004; Jung et al. 2007).

Chitosan is a polycationic biopolymer naturally present in the exoskeletons of crustaceans and arthropods (Tikhonov et al. 2006). Partially and fully deacetylated chitosan derivatives of low molecular weight are available, with broad antibacterial and antifungal activity (Franklin and Snow 1981; Kong et al. 2010). Chitosan is considered a safe food additive. Reported antibacterial activity for chitosan

derivatives include both Gram-positive and Gram-negative bacteria such as *S. aureus, L. monocytogenes, B. cereus, E. coli, Shigella dysenteria*e, and *Salmonella* Typhimurium (Gyawali and Ibrahim 2014). Chitosan has attracted great attention for development of biodegradable edible coatings, singly or dosed with other antimicrobial substances (Maher et al. 2013).

2.3 Antimicrobials Derived from Plants

Herbs and spices have been recognized to possess a broad spectrum of active constituents that exhibit antibacterial, antifungal, antiparasitic, and/or antiviral activities. Essential oils have been used for centuries as part of natural traditional medicine. They are aromatic oily liquids obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots). The major groups of principal components that make essential oils effective antimicrobials include saponins, flavonoids, carvacrol, thymol, citral, eugenol, linalool, terpenes, and their precursors (Burt 2004). The antimicrobial activity of alliums is mainly attributed to various kinds of alk(en)yl alka/ene thiosulfinates (thiosulfinates; and their transformation products (Kyung 2012). Allium-derived antimicrobial compounds inhibit microorganisms by reacting with the sulfhydryl (SH) groups of cellular proteins. In olive oil, distinctive antimicrobial compounds including oleuropein, oleuropein aglycon, elenoic acid and oleocanthal (in addition to hydroxytyrosol and tyrosol) have been described (Cicerale et al. 2012). The antibacterial activities of essential oils and other plant extracts has attracted great attention for application of the crude extracts or their bioactive components in food biopreservation (Burt 2004; Holley and Patel 2005; Richard and Patel 2005; Tajkarimi et al. 2010). In the concentration range of 0.05–0.1 %, essential oils have demonstrated activity against pathogens, such as *S.* Typhimurium, *E. coli* O157:H7, *L. monocytogenes*, *B. cereus* and *S. aureus*, in food systems. However, activity against various microorganisms on food products might be higher than the concentration applied for flavoring purposes. As a result, this might cause food tainting and/or adverse sensorial effects to food products (Bagamboula et al. 2004). It has been suggested that the adverse sensorial effects of essential oils agents to food products can be overcome by masking the odor with other approved aroma compounds (Gutiérrez et al. 2009). Antimicrobial agents derived from essential oils are interesting candidates for development of activated films or packagings.

Plants also produce a variety of antimicrobial peptides, many of which can be grouped in different classes: thionins, defensins, lipid transfer proteins, cyclotides and snakins (Padovan et al. 2010). Some of them could possibly be exploited for food biopreservation. Interestingly, plants can be a good source of antifungal proteins and peptides, including chitinases, glucanases, thaumatin-like proteins, thionins, and cyclophilin-like proteins (Ng 2004).

2.4 Bacteriophages

Bacteriophages are obligate parasites of bacteria. Lytic bacteriophages offer a great potential as natural biopreservative agents, due to their capacity to selectively control bacterial populations. This phenomenon occurs spontaneously in Nature, but can also be applied purposedly in food systems. One feature of bacteriophages is their high host specificity, at the level of species and even strains. Specificity at strain level can be a limitation for application of bacteriophages. Nevertheless, several studies have shown the efficacy in food systems of mixtures containing different bacteriophages and also broad-host range bacteriophages that were able to attack a high number of bacterial strains, including the most virulent strains found in foods (Hagens and Loessner 2007, 2010, 2014; Sharma 2013; Sulakvelidze 2013). Control of *L. monocytogenes* by bacteriophages has been addressed in many different ready-to-eat foods of animal as well as plant origin. Commercial phage preparations like ListShield™ (containing a mixture of six naturally occurring listeriophages) and Listex™ P100 (based on listeriophage P100) have been approved by the FDA and USDA (Hagens and Loessner 2014). Bacteriophages specific for *Salmonella* serotypes have also been used on various food substrates such as sprouted seeds and animal skins and carcasses. The commercial preparation SalmoFresh contains a cocktail of naturally occurring lytic bacteriophages that selectively and specifically kill *Salmonella*, including strains belonging to the most common/highly pathogenic serotypes: Typhimurium, Enteritidis, Heidelberg, Newport, Hadar, Kentucky, and Thompson. Bacteriophages specific for *E. coli* (including virulent strains) have also shown efficacy on different food substrates. The commercial preparation EcoShield™ contains a cocktail of three lytic phages specific for *E. coli* O157:H7. Illustrative studies on application of bacteriophages can be found in the scientific literature for other human pathogenic or toxinogenic bacteria such as *Shigella* spp. (Zhang et al. 2013), *C. jejuni* (Bigwood et al. 2008), *Cronobacter sakazakii* (Zuber et al. 2008), *S. aureus* (Bueno et al. 2012), as well as spoilage bacteria such as *Pseudomonas fluorescens* (Sillankorva et al. 2008). *Brochothrix thermosphacta* (Greer and Dilts 2002) or *Leuconostoc gelidum* (Greer et al. 2007).

Another emerging field of interest is the application of bacteriophages for reducing the carriage of zoonotic agents in livestock and poultry and also for the prophilaxy and therapy in diseased animals. Phage therapy is potentially useful in virulent *Salmonella* and *E. coli* infections in chickens, calves and pigs, and in control of the food-borne pathogens *Salmonella* and *C. jejuni* in chickens and *E. coli* O157:H7 in cattle (Johnson et al. 2008; Connerton et al. 2011; Sulakvelidze 2013; Endersen et al. 2014). Selective application of bacteriophages could improve animal health and animal production and reduce the risks of transmission of zoonotic agents to humans.

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Chapter 3
Application of Lactic Acid Bacteria and Their Bacteriocins for Food Biopreservation

3.1 Bacteriocins and Bactericin-Producing Strains

Microbes and/or their natural products have played key roles in the preservation of foods during mankind history (Ross et al. 2002). The rational exploitation of microbial antagonism based on scientific knowledge has been possible after the discovery of the biochemical nature of the antimicrobial substances produced by microorganisms. Bacteriocins produced by the lactic acid bacteria (LAB) have several features that still make them attractive for food preservation: (1) LAB have a long history of safe use in foods; (2) LAB and their cell products—including bacteriocins—are generally recognised as safe; (3) LAB bacteriocins are not active and non-toxic on eukaryotic cells, and (4) due to their proteinaceous nature, bacteriocins are expected to become inactivated by digestive proteases and not exert significant effects on gut microbiota at the concentrations ingested with the food. In addition, LAB bacteriocins may be suitable as preservatives, given (1) their sometimes broad antimicrobial spectrum, including food poisoning and spoilage bacteria, (2) their synergistic activity with other antimicrobials, (3) a bactericidal mode of action exerted at membrane level, which avoids cross resistance with antibiotics of clinical use, (4) stability under the heat and pH conditions achieved under processing of many foods, and (5) their genetic determinants are usually plasmid-encoded, which facilitates genetic manipulation and development of producer strains with improved technological properties. Bacteriocin-encoding plasmids may be transferred to other strains by natural processes, but at the same time there is a risk for loss of the plasmid together with the bacteriocin production capacity.

Application of bacteriocins in food preservation may be beneficial in several aspects (Thomas et al. 2000; Gálvez et al. 2007): (1), to decrease the risks of food poisoning; (2) to decrease cross contamination in the food chain; (3) improve the

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shelf life of food products; (4) food protection during temperature abuse episodes; (5) decrease economic losses due to food spoilage; (6) reduce the levels of added chemical preservatives; (7) reduce the intensity of physical treatments, achieving a better preservation of the food nutritional value and possibly decrease of processing costs; (8) may provide alternative preservation barriers for "novel" foods (less acidic, with a lower salt content, and with a higher water content), and (9) may satisfy the demands of consumers for foods that are fresh-tasting, lightly-preserved, and ready to eat (RTE). There may also be a potential market for bacteriocins as natural substitutes for chemical preservatives, and in the preservation of functional foods and nutraceuticals (Robertson et al. 2004).

According to previous studies (Deegan et al. 2006; Gálvez et al. 2007), bacteriocins can be applied in foods in many different ways:

(a) Addition of bacteriocin preparations. These are often obtained by cultivation of the producer strains in growth media suitable for bacteriocin production. The bacteriocin production step is usually followed by inactivation of the producer bacterial cells (for example by heat or UV treatments) and concentration of the cultured broths (by lyophilisation or spray-drying) to obtain a bio-active powder which contains a mixture of the antimicrobial substances produced in broth (such as the bacteriocin and organic acids). Commercial preparations such as Nisaplin™, Alta™ products or Microgard™ are some examples. Other bacteriocins such as lacticin 3147, variacin from *Kokuria varians* or enterocin AS-48 have also been obtained as dry powder preparations (Morgan et al. 1999; O'Mahony et al. 2001; Ananou et al. 2010b). However, most bacteriocins have only been produced on laboratory culture media, and recovered as partially purified concentrates by standard protein purification techniques such as ammonium sulphate precipitation or ion exchange chromatography.

(b) Bacteriocin-producing cultures. These may come in the form of lyophilized preparations for propagation and inoculum build up or direct addition to the food, as well as overnight cultures or even refrigerated cell concentrates. Bacteriocin-producing cultures must contain metabolically active bacterial cells ready for propagation in the food substrate and able to carry a rapid in situ bacteriocin production. Bacteriocin-producing strains can be applied as the main starter cultures in fermented foods provided that they offer the technological properties required for the fermentation, or as an adjunct culture in combination with bacteriocin-resistant starter strains. They can also be applied as bio-protective cultures in non-fermented foods, provided that they do not have adverse effects on the food. For application in foods stored under refrigeration, a desirable property would be the capacity to produce bacteriocin at low temperature. In some cases, a low bacteriocin production during refrigeration storage can be compensated with the simultaneous addition of a bacteriocin concentrate together with producer culture.

3.2 Application of LAB Bacteriocins as Part of Hurdle Technology

The efficacy of bacteriocins can improve considerably when applied in combination with other antimicrobials or barriers. As a matter of fact, food preservation most often relies on application of several barriers that restrict the survival and proliferation of microorganisms. These include treatments for inactivation of microorganisms in the raw materials, during processing, or in the finished product, acidification, addition of preservatives, and/or modification of atmosphere composition among others. The concept of hurdle technology (Leistner 2000) is based on the combination of different barriers acting in different ways on microbial cells, so that the cells have to activate different repair and adaptation mechanisms in order to survive and/or proliferate under the imposed selective conditions. Under such varied selective pressure, the cells will die as a consequence of energy exhaustion and failure to repair cell damages. Since most bacteriocins act on the bacterial cytoplasmic membrane, they interfere with the generation of energy required to repair bacterial cell damage. At the same time, cell damage or metabolic constraints imposed by other hurdles reduce the natural defense mechanisms of bacteria, making them susceptible at low bacteriocin concentrations that would not be lethal to intact cells. Some of the hurdles may also destabilize bacterial cell structures such as the outer membrane of Gram-negative bacteria. The outer membrane acts as a selective permeability barrier that retains bacteriocins and other antimicrobial substances. When this barrier is destabilized, bacteriocins (as well as other antimicrobials) can diffuse much better and reach the bacterial cytoplasmic membrane, where they act specifically.

The scientific literature is full of examples where bacteriocins have been tested in different food systems as part of hurdle technology, with several purposes (Gálvez et al. 2007, 2008): (1) enhancing the efficacy of bacteriocins as well as that of treatments; (2) reducing the required bacteriocin concentration; (3) broadening the spectrum of antimicrobial treatments (for example, to Gram-negative bacteria); (4) improving the inactivation of bacterial endospores; (5) as an additional barrier to proliferation of sublethally-injured cells as well as intact cells and endospores surviving treatments; and (6) as an additional barrier against post-process contamination. Specific examples of these applications will be discussed in the following chapters dealing with application of bacteriocins in different food systems. Notwithstanding, a summary of combined treatments and their reported effects is presented in Table 3.1.

Table 3.1 Application of bacteriocins in combined treatments

Antimicrobial treatment	Reported effects	Reference(s)
CO_2 atmosphere	Synergism with bacteriocins against foodborne pathogens (nisin, pediocin PA-1/Ach)	Nilsson et al. 2000; Szabo and Cahill 1999
Nitrates, nitrites	Enhanced antibacterial activity of nisin in meats against *C. botulinum*, *Ln. mesenteroides* and *L. monocytogenes*	Rayman et al. 1983; Gill and Holley 2003
Organic acids and salts	Improve bacteriocin solubility and increase sensitivity of target bacterial cells in different food substrates (nisin, pediocin PA-1/AcH, enterocin AS-48, or lacticin 3147…)	Scannell et al. 1997, 2000; Uhart et al. 2004; Cobo Molinos et al. 2005
	Sensitize Gram-negative bacteria due to their chelating activity	Scannell et al. 1997
Chelating agents (EDTA, polyphosphates)	Destabilization of the outer bacterial cell membrane in Gram-negative bacteria and improving cell permeability to bacteriocins	Stevens et al. 1991; Vaara 1992; Helander et al. 1997
	Sequestration of essential mineral nutrients and indirect sensitization to bacteriocins on Gram-positive bacteria	Gill and Holley 2000, 2003
Essential oils and their bioactive components	Greater inactivation of Gram-positive bacteria and sensitization of Gram-negative bacteria to bacteriocins	Pol et al. 2001a; Yuste and Fung 2004; Grande et al. 2007; Cobo Molinos et al. 2009
Other antimicrobials of chemical or proteinaceous nature[a]	Increased inactivation of Gram-positive and/or Gram-negative bacteria	Mulet-Powell et al. 1998; Gill and Holley 2000; Nattress and Baker 2003; Branen and Davidson 2004; Boussouel et al. 2000; Naghmouchi et al. 2010; Lüders et al. 2003
CO_2 atmosphere	Inhibition of strictly aerobic bacteria (complementary action with bacteriocins)	Economou et al. 2009
Heat	Increased inactivation of Gram-positive bacteria	Budu-Amoako et al. 1999; Ananou et al. 2004
	Perturbation of the bacterial outer cell membrane and sensitization of Gram-negative bacteria to bacteriocins	Kalchayanand et al. 1992; Boziaris et al. 1998; Ananou et al. 2005; Bakes et al. 2004
	Activation of endospore germination and reduced heat resistance of bacterial endospores	Wandling et al. 1999; Grande et al. 2006
	Additional barrier against endospores after heat treatments	Wandling et al. 1999; Grande et al. 2006

High intensity pulsed electric fields	Synergistic to additive effects on Gram-positive bacteria	Calderón-Miranda et al. 1999; Sobrino-Lopez and Martin Belloso 2006; Pol et al. 2001b
	Sensitization of Gram-negative bacteria, thus improving microbial inactivation in the combined treatments	Liang et al. 2002; Terebiznik et al. 2000; Martínez-Viedma et al. 2008
	Additional barrier against proliferation of survivors and sublethally-injured cells during the product shelf life	Martínez-Viedma et al. 2009
High hydrostatic pressure	Improved inactivation of Gram-positive bacteria	Ananou et al. 2010a; Ponce et al. 1998; Capellas et al. 2000; Arqués et al. 2005; López-Pedemonte et al. 2003
	Sensitization of Gram-negative bacteria, thus improving microbial inactivation in the combined treatments	Ponce et al. 1998; Garcia-Graells et al. 1999; Black et al. 2005; Masschalck et al. 2001
	Additional barrier against proliferation of survivors and sublethally-injured cells during the product shelf life	López-Pedemonte et al. 2003; Arqués et al. 2005; Marcos et al. 2008
High pressure homogeneization	Improved inactivation of *L. monocytogenes* in carrot juice	Pathanibul et al. 2009
Irradiation	Improved inactivation of *L. monocytogenes,* and protection against post-process contamination of the food	Chen et al. 2004
Pulsed light	Improved inactivation of *L. monocytogenes,* and protection against post-process contamination of the food	Uesugi and Moraru 2009

[a]lysozyme, lactoferrin, ovalbumin, lactoperoxidase system, and other antimicrobial peptides

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Chapter 4
Biopreservation of Meats and Meat Products

4.1 Application of Bacteriocin Preparations

4.1.1 Raw Meats

The microbial populations most frequently associated with the meat environment are known to primarily belong to the groups Enterobacteriaceae, lactic acid bacteria (LAB), *Brochothrix thermosphacta*, and pseudomonads (Borch et al. 1996; Labadie 1999; Nychas et al. 2008). Microbial metabolism of meat during growth results in microbial spoilage, with the development of offodors which make the product undesirable for human consumption (Jackson et al. 1997). Also, pathogenic bacteria initially present at low concentrations may grow during meat spoilage may proliferate during refrigeration storage, especially *Listeria monocytogenes*.

In raw meats, bacteriocins have been tested alone or in combination with other hurdles for carcass decontamination and/or to inhibit bacterial growth on stored fresh meats (Table 4.1). Washing, spraying or dipping with bacteriocin solutions have been tested alone or in combination with other antimicrobials to potentiate bacteriocin activity. In order to increase the efficacy of treatments and/or avoid cross contamination, raw meats are chilled, packaged under different atmospheric conditions such as vacuum packaging, MAP, or active packaging with O_2 scavengers or CO_2 generating systems (Coma 2008; McMillin 2008). Additional combinations such as low dose irradiation, UV surface decontamination or HHP have been proposed (Aymerich et al. 2008). All these processing treatments have selective effects the initial microbiota, and may act in synergy with bacteriocins to increase the product safety and shelf life. Although raw meat products are further processed prior to consumption by treatments that usually destroy pathogenic bacteria, they can be a considerable source of cross contamination. Growth of toxin-producing bacteria in raw materials (such as minced meats) should also be controlled, especially for heat-stable toxins.

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Table 4.1 Examples of applications of bacteriocin preparations in meat and poultry products

Bacteriocin preparations	Effect(s)	Reference(s)
Raw meats		
Nisin combinations (organic acids, chelators, lysozyme, vacuum packaging, MAP)	Decontamination of raw meat surfaces before processing	Thomas et al. 2000
Nisin activated film with EDTA	Inhibition of LAB, carnobacteria and *B. thermosphacta* and reduction of *Enterobacteriaceae* load on beef cuts	Ercolini et al. 2010
Pediocins	Anti-listeria protection by pediocins in raw meats	Rodríguez et al. 2002
Pentocin 31-1	Reduction of growth of *Listeria* and *Pseudomonas* and total volatile basic nitrogen production in chill-stored tray-packaged pork meat	Zhang et al. 2010
RTE meats		
Nisin activated films	Increased inactivation of *L. monocytogenes* in several vacuum-packaged products	Aymerich et al. 2008
Nisin in combination with HHP	Increased inactivation of *E. coli* and staphylococci in cooked ham, avoiding regrowth of *E. coli* and slime-forming bacteria	Garriga et al. 2002
Nisin and pulsed light	Application of a Nisaplin dip followed by exposure to pulsed light reduced the population of *L. innocua* on sausages	Uesugi and Moraru 2009
Nisin-pectin film, in combination with low-dose irradiation	Increased microbial inactivation of *L. monocytogenes* on RTE turkey meat and inhibition of survivor proliferation during storage	Jin et al. 2009
Pediocin in combination with post-packaging irradiation or thermal treatment	Effective combination with to control *L. monocytogenes* on frankfurters	Chen et al. 2004a, b
Enterocin alginate film, in combination with HHP	Prevention of *L. monocytogenes* regrowth in the treated cooked ham during cold storage as well as during cold chain break	Marcos et al. 2008a, b

At present, there is a great body of research data concerning bacteriocin trials on raw meats, many of them dealing with nisin. Nisin has been widely tested for preservation of raw meats (Thomas et al. 2000). However, the application of nisin in meats has several drawbacks such as its poor solubility, interaction with phospholipids and antagonism by glutathione (Thomas et al. 2000; Stergiou et al. 2006). Nevertheless, positive results have been reported for surface decontamination of raw meats before processing and packaging, in which antimicrobial activity was potentiated by combination with other antimicrobials or hurdles such as organic acids, chelators, vacuum packaging, or MAP. Representative examples reported on vacuum-packaged beef are the reduction in the numbers of *Listeria innocua* and *B. thermosphacta* after nisin treatment (Cutter and Siragusa 1996a) or the inhibition of *L. monocytogenes* and *Escherichia coli* O157:H7 after treatment with nisin and

EDTA (Zhang and Mustapha 1999). Similarly, dipping in solutions containing combinations of lactic or polylactic acids and nisin reduced the microbial load of meats before processing and afforded an extended shelf-life in vacuum-packaged fresh meat (Ariyapitipun et al. 1999, 2000; Barboza de Martinez et al. 2002), and treatment with a combination of nisin and lysozyme effectively inhibited *B. thermosphacta* and LAB in vacuum-packaged pork (Nattress et al. 2001; Nattress and Baker 2003). Other reports indicated that, under MAP, nisin was able to completely inhibit growth of *L. monocytogenes* in pork (Fang and Lin 1994a, b).

In raw poultry meats, application of antimicrobial treatments using nisin and EDTA to in combination with MAP or vacuum packaging (VP) reduced total aerobic plate counts and increased the product shelf-life by a minimum of 4 days when packaged under aerobic conditions and a maximum of 9 days when vacuum packaged (Cosby et al. 1999). The use of MAP (65 % CO_2, 30 % N_2, 5 % O_2) in combination with nisin–EDTA antimicrobial treatments affected the populations of mesophilic bacteria, *Pseudomonas* sp., *B. thermosphacta*, lactic acid bacteria and *Enterobacteriaceae*, and resulted in an organoleptic extension of refrigerated, fresh chicken meat for up to 14 days, decreasing the formation of volatile amines, trimethylamine nitrogen and total volatile nitrogen (Economou et al. 2009).

Treatment of raw meats and poultry meats with pediocins (especially pediocin PA-1/Ach) singly or in combination with other hurdles can inhibit or delay growth of spoilage Gram-positive bacteria (such as *B. thermosphacta*) and/or reduce *L. monocytogenes* populations (Rodríguez et al. 2002; Nieto-Lozano et al. 2006; Kalchayanand 1990; Nielsen et al. 1990; Motlagh et al. 1992; Degnan et al. 1993; Schlyter et al. 1993: Taalat et al. 1993; Goff et al. 1996; Murray and Richard 1997). For example, treatment of raw meat surfaces with 500, 1,000 or 5,000 bacteriocin units/ml (BU/ml) reduced the counts of inoculated *L. monocytogenes* after storage at 15 °C during 72 h by 1, 2 or 3 log cycles, and treatment with 1,000 or 5,000 BU/ml reduced its viable counts by 2.5 or 3.5 log cycles, respectively, after storage at 4 °C during 21 days compared to the control not treated with bacteriocin. The same bacteriocin treatments exerted a bacteriostatic effect on *Clostridium perfringens* (Nieto-Lozano et al. 2006). In poultry meats, treatment with pediocin PA-1/Ach adsorbed to heat killed *Pediococcus acidilactici* cells was very effective in the control of *L. monocytogenes* in refrigerated chicken meat (Goff et al. 1996).

Other bacteriocins such as sakacins, carnobacteriocins, bifidocins, lactocins, lactococcins, enterocins or pentocins have shown variable inhibitory effects against spoilage or pathogenic bacteria in raw meats or poultry meats (Aymerich et al. 2000, 2008; Galvez et al. 2008). In chicken breasts, addition of enterocins A and B produced by the meat isolate *Enterococcus faecium* CTC492 (4,800 AU/cm^2) reduce the population of *Listeria* to 3.6 MNP/cm^2 during incubation at 7 °C (Aymerich et al. 2000). In vacuum-packed chicken cuts stored under refrigeration, treatment with sakacin-P caused strong inhibition of *L. monocytogenes* (Katla et al. 2002). Addition of bifidocin B (from *Bifidobacterium bifidum*) and lactococcin R (produced by *Lactococcus lactis* subsp. *cremoris*) to irradiated raw chicken breast inhibited the growth of *L. monocytogenes* or *Bacillus cereus* for 3–4 weeks at 5–8 °C or 6–12 h at 22–25 °C (Yildirim et al. 2007). Another study reported that application

of pentocin 31-1 (produced by a *Lactobacillus pentosus* strain isolated from the traditional Chinese fermented Xuan-Wei Ham) in chill-stored non-vacuum tray-packaged pork meat substantially reduced the growth of *Listeria* and *Pseudomonas* as well as the total volatile basic nitrogen (measured as an indicator of meat spoilage) during cold storage compared with the untreated control (Zhang et al. 2010).

One attractive approach to optimize the activity of bacteriocins in raw meats has been immobilisation in substrates (such as beads, liposomes, coatings or films). Nisin (alone or in combinations with citric acid, EDTA, and Tween 80) incorporated in a variety of substrates (such as calcium alginate gels, agar coatings, palmitoylated alginate-based films, polyvinyl chloride, LDPE, or nylon) showed strong inhibition of bacteria such as *L. monocytogenes*, *B. thermosphacta*, *Staphylococcus aureus*, or *Salmonella* Typhimurium on refrigerated raw meats (Chen and Hoover 2003; Aymerich et al. 2008; Gálvez et al. 2007, 2008). This approach decreases the impact of interaction with food components and enzyme inactivation of bacteriocin activity, and also decreases the amount of bacteriocin required for inhibition of target bacteria (Quintavalla and Vicini 2002). In raw meats and poultry samples packaged in bags coated with pediocin powder, the pediocin completely inhibited growth of inoculated *L. monocytogenes* through 12 weeks storage at 4 °C (Ming et al. 1997). Application of nisin immobilized in calcium alginate gel on beef carcass tissues completely suppressed *B. thermosphacta* (Cutter and Siragusa 1996b), and low density polyethylene films containing nisin prevented carcass contamination by this bacterium (Siragusa et al. 1999). Nisin bound to activated alginate beads or in a palmitoylated alginate-based film (to avoid nisin degradation) reduced the viable counts of *S. aureus* in ground beef and on sliced beef meat, respectively (Millette et al. 2007). Treatment of fresh poultry with agar coatings containing nisin achieved substantial reductions in *S.* Typhimurium growth after storage at 4 °C for 96 h (Natrajan and Sheldon 1995). The efficiency of numerous films formulation based on polyvinyl chloride, LDPE, nylon, calcium-alginate or agar containing nisin (in combinations with citric acid, EDTA, and Tween 80) to inhibit the antibiotic resistant *S.* Typhimurium on poultry drumstick skin was demonstrated by the same researchers (Natrajan and Sheldon 2000a, b). Combinations of nisin with citric acid, EDTA and Tween-80 also led to a 4–5 log reduction of psychotrophic aerobes during 72 h of storage. In another study, Ercolini et al. (2010) tested a nisin activated plastic antimicrobial packaging (developed by using a nisin, HCl and EDTA solution) on beef cuts stored at 1 °C. The combination of chill temperature and antimicrobial packaging proved to be effective in enhancing the microbiological quality of beef cuts by inhibiting LAB, carnobacteria and *B. thermosphacta* in the early stages of storage and by reducing the loads of *Enterobacteriaceae*, without affecting the species diversity according to PCR-DGGE fingerprints of DNA extracted from the treated meat cuts (Ercolini et al. 2010). Also, plastic bags activated at their internal face with a nisin-EDTA solution were used for vacuum-packaging of beef chops (Ferrocino et al. 2013). During storage in the activated films at 1 °C, *B. thermosphacta* was unable to grow for the whole storage time (46 days), while the levels of *Carnobacterium* spp. were below the detection limit for the first 9 days and reached levels below 5 log CFU/cm^2 after 46 days. The antimicrobial packaging had no

effect on *Enterobacteriaceae* or *Pseudomonas* spp., with final populations of about 4 log CFU/cm^2. Nevertheless, the active packaging reduced the release of volatile metabolites in the headspace of beef with a probable positive impact on meat quality. Recycling of industrial wastes into useful products is a growing trend not only in the food industry but in many other fields as well. In a recent and innovative study, a novel poly(lactic acid)/sawdust particle biocomposite film with anti-listeria activity was developed by incorporation of pediocin PA-1/AcH (Woraprayote et al. 2013). It was reported that sawdust particle played an important role in embedding pediocin into the hydrophobic PLA film. Application of the activated film as a food-contact antimicrobial packaging on raw sliced pork efficiently inhibited *L. monocytogenes* during chill storage.

The bacteriocin 32Y (from *Lactobacillus curvatus* 32Y) was used to develop an industrially produced activated plastic film (Mauriello et al. 2004). In experiments of food packaging with pork steak and ground beef (simulating hamburgers) contaminated by *L. monocytogenes* V7, highest antimicrobial activity was observed after 24 h at 4 °C, with a decrease of about 1 log of the *L. monocytogenes* population (Mauriello et al. 2004). The lactocins 705 and AL705 are produced by *L. curvatus* CRL705. Lactocin 705 has antagonist effect against LAB and *B. thermosphacta*, while AL705 is active against *Listeria* species (Castellano and Vignolo 2006). Both bacteriocins retained antimicrobial activity when included in polymer matrices such as LDPE (Blanco et al. 2008, 2012) and gluten (Blanco Massani et al. 2014). In trials with *L. curvatus* CRL705 immobilized bacteriocins, a bacteriostatic effect against *L. innocua* 7 was observed in both synthetic (Cryovac films) and gluten activated packages until the fourth week of storage (Blanco Massani et al. 2014).

The process operations for manufacture of minced meats facilitate inoculation of contaminating bacteria in the meat batter. Therefore, the presence and multiplication of foodborne pathogens in minced meats should be controlled. One study showed that the single addition of nisin extended the lag phase of *L. monocytogenes* inoculated into minced buffalo meat (Pawar et al. 2000). In minced meats, the combination of bacteriocins with plant essential oils at levels where they would not impart undesirable flavour is being considered as a way to increase inactivation of *L. monocytogenes* and inhibition of *Salmonella* Enteritidis (Solomakos et al. 2008; Govaris et al. 2010). Antilisterial activity of nisin in minced beef increased greatly in combination with thyme essential oil. The combination of essential oil at 0.6 % with nisin at 1,000 IU/g decreased the population of *L. monocytogenes* below the official limit set by European Union during storage at 4 °C for at least 12 days (Solomakos et al. 2008). At that concentration, the thyme oil did not impart undesirable flavour. Promising results have also been reported on inhibition of *S*. Enteritidis in sheep minced meat by a combination of nisin and oregano essential oil (Govaris et al. 2010), while the single treatment of minced sheep meat with nisin at 500 or 1,000 IU/g had no activity against *S*. Enteritidis. The combination of the oregano essential oil at 0.6 % with nisin at 500 IU/g showed stronger antimicrobial activity against *S*. Enteritidis than the single oregano essential oil at 0.6 % but lower than the combination with nisin at 1,000 IU/g (Govaris et al. 2010). Best results were reported for the combinations of oregano essential oil at 0.9 % with nisin at 500 or

1,000 IU/g, which showed a bactericidal effect against the pathogen. The inhibitory effects were higher in samples stored at 10 °C compared to 4 °C. This could be a draw-back for cold-stored meats, but at the same time could be an advantage under episodes of cold chain break and temperature abuse. Regarding the effects of other bacteriocins in minced meats, addition of a partially-purified plantaricin preparation from *Lactobacillus plantarum* UG1 rapidly reduced the population of *L. monocytogenes* below detectable levels in minced meat stored at 8 °C, (Enan et al. 2002), and the addition of a freeze-dried whey fermentate from *C. piscicola* (containing piscicocin CS526) to a ground mixture of beef and pork meat reduced the population of *L. monocytogenes* below detectable levels for at least 4 days at 12 °C and for up to 25 days at 4 °C (Azuma et al. 2007). Furthermore, in minced pork treated with a preparation of enterocins A and B (1,600 AU/g) from *E. faecium* CTC492, the levels of listeria were reduced below 3 MNP/g after 6 days of incubation at 7 °C while the untreated control increased from 5 MNP/g to 48 CFU/g (Aymerich et al. 2000).

4.1.2 Semi-processed and Cooked Meats

Cooked meat products are widely consumed ready-to-eat (RTE) foods. They may consist of whole primary meat pieces, but usually they are made by grinding and mixing secondary meats, fat, animal organs, or blood with other ingredients, followed by stuffing/molding and cooking. The cooking process inactivates natural microbiota, paving the way for growth of post-process contaminants. The pH values of most cooked meat products are compatible with growth of pathogenic and spoilage bacteria, which can proliferate at refrigeration temperatures during the product shelf life. Some of these meats may also undergo further processing such as slicing, peeling, and packaging, which increase the risks for cross-contamination (Murphy et al. 2005). For these reasons, there has been a great interest in the application of bacteriocins (mainly pediocin and nisin) as hurdles against spoilage bacteria and pathogens (mainly *L. monocytogenes*). The main approaches tested are based on addition of bacteriocin preparations to the meat slurries before the heating process, surface application of the bacteriocins before packaging, or application of films or coatings dosed with bacteriocins. The possibility of adding bacteriocins in the meat before the cooking process due to their thermotolerance is of great interest.

Strains of LAB (mainly *Lactobacillus* and *Leuconostoc*) are the major group of spoilage bacteria developing on various types of vacuum-packed meats, where they produce typical sensory changes such as souring, gas, SH_2 and slime (Korkeala et al. 1988; Björkroth and Korkeala 1997). In one study using sakacin K, nisin and enterocins, the results obtained clearly depended on the bacteriocin and the target bacteria (Aymerich et al. 2002). Sakacin K and nisin were unable to prevent ropiness caused by *Lactobacillus sakei* CTC746 strain, but nisin was able to prevented ropiness caused by *Leuconostoc carnosum* CTC747 (Aymerich et al. 2002). Nisin was also the most effective bacteriocin on staphylococci, but did not prevent regrowth of *L. monocytogenes* (while enterocins, sakacin and pediocin did).

Ovotransferrin is the main component in the antimicrobial defense system of hens' egg. Antimicrobial activity of ovotransferrin is mainly due to its iron-binding capacity, but direct interactions with the bacterial surface also seem to play an important role in contributing to its inhibitory activity (Moon et al. 2011). Ovotransferrin, nisin, and their combinations had strong antilisterial activity in BHI broths. However, addition of ovotransferrin to frankfurters did not inhibit growth of *L. monocytogenes*. When nisin (1,000 IU/frankfurter) was applied, an early bactericidal effect followed by delayed growth was observed (Moon et al. 2011). However, no differences were reported in the antilisterial effect when the same nisin concentration was applied in combination with ovotransferrin (40 mg/frankfurter). The observed differences could be explained by the influence of factors such as interaction with food substrate or a higher iron content in meat.

Incorporation of nisin into bologna-type sausages during mixing of ingredients inhibited the growth of spoilage LAB during further storage at 8 °C of the resulting vacuum-packed sausages (Davies and Delves-Broughton 1999). The effectiveness of nisin against several bacteria (such as *B. thermosphacta*, *L. curvatus*, *Ln. mesenteroides*, *L. monocytogenes*, *Salmonella* sp. and *E. coli* O157:H7) in ham and/or bologna sausages increased in combination with lysozyme and EDTA (Gill and Holley 2000a, b). In fresh pork sausages, a combination of nisin and organic acids reduced the viable counts of *Salmonella* Kentucky and *S. aureus* (Scannell et al. 1997). The combination of sodium citrate or sodium lactate with nisin or lacticin 3147 was also reported to increase the inhibition of *Listeria* and *C. perfringens* in fresh pork sausages (Scannell et al. 2000a).

Pediocin activity was increased when added in combinations with sodium diacetate or sodium lactate against *L. monocytogenes* on frankfurters or *L. monocytogenes* and *Yersinia enterocolitica* on cooked poultry cuts stored under MAP at 3.5 °C (O'Sullivan et al. 2002; Chen and Hoover 2003; Aymerich et al. 2008). The antilisterial activity of pediocin in slurries prepared from ready-to-eat turkey breast meat increased greatly when tested in combination with diacetate, due to synergistic effects between the two antimicrobials (Schlyter et al. 1993). When commercial beef franks were dipped for 5 min in three antimicrobial solutions: pediocin (6,000 AU), 3 % sodium diacetate and 6 % sodium lactate combined, and a combination of the three antimicrobials, reductions of *L. monocytogenes* populations ranged between 1 and 1.5 log units and 1.5–2.5 log units after 2 and 3 weeks of storage, respectively, at 4 °C (Uhart et al. 2004). These results indicated that the use of combined antimicrobial solutions for dipping treatments is more effective at inhibiting *L. monocytogenes* than treatments using antimicrobials such as pediocin separately (Uhart et al. 2004). In another study, the effects and interactions of temperature (56.3–60 °C), added sodium lactate (0–4.8 %) and sodium diacetate (0–0.25 %) and dipping in pediocin (0–10,000 AU) on *L. monocytogenes* in bologna were studied by Maks et al. (2010). Combination treatments increased or decreased *D*-values, depending on the temperature. Pediocin (2,500 and 5,000 AU) and heat decreased *D*-values, but pediocin exhibited a protective effect at higher concentrations (≥7,500 AU). The results showed that interactions between additives in formulations can vary at different temperatures/concentrations, thereby affecting thermal inactivation of foodborne pathogens in meat products.

Enterocins have also been tested in cooked meat products. Addition of a partially-purified preparation of enterocins A and B (4,800 AU/g) reduced the numbers of *L. innocua* by 7.98 log cycles in cooked ham and by 9 log cycles in pork liver paté stored at 7 °C for 37 days (Aymerich et al. 2000). In vacuum packaged sliced cooked pork ham, added enterocins A and B (128 AU/g) inhibited the production of slime by *Lactobacillus sakei* CTC746 strain, but not by *Leuconostoc carnosum* CTC747 strain (Aymerich et al. 2002).

Results from studies on the synergistic activities of bacteriocins with other antimicrobials and on the effect of immobilized preparations or application of bacteriocins by dipping solutions, together with the technical advances in the development of activated supports opened the doors for application of immobilized bactericin preparations or activated packagings containing cocktails of antimicrobial substances on RTE meats (Coma 2008). Bacteriocin-activated films may be quite useful for cooked meat products, not only because they can prolong the product shelf life by decreasing the risks of spoilage and growth of pathogens from cross-contamination during processing, but also because the film itself acts as a barrier against external contamination of the processed product. Among the various kinds of edible coatings tested on vacuum-packaged products (hot dogs, frankfurters, or ham) best results have been reported for coatings containing nisin in combination with other antimicrobials under refrigeration storage.

Application of zein coatings containing nisin, sodium lactate, and sodium diacetate completely eliminated *L. monocytogenes* on turkey frankfurters during refrigeration storage (Lungu and Johnson 2005). In hot dogs that were vacuum-packaged in films coated with nisin, *L. monocytogenes* counts decreased during refrigeration storage (Franklin et al. 2004). Hot dogs were placed in control and nisin-containing pouches and inoculated with a five-strain *L. monocytogenes* cocktail (approximately 5 log CFU per package), vacuum sealed, and stored for intervals of 2 h and 7, 15, 21, 28, and 60 days at 4 °C. In hot dogs packaged in films coated with 2,500 IU/ml nisin solution, nisin significantly decreased ($P<0.05$) *L. monocytogenes* populations on the surface of hot dogs by greater than 2 log CFU per package throughout the 60-days study. However, *L. monocytogenes* populations still remained at approximately 4 log CFU per package after 60 days of refrigerated storage (Franklin et al. 2004). This study reported similar results when using a cellulose-based coating solution (based on methylcellulose/hydroxypropyl methylcellulose) containing nisin. However, in another study nisin-coated cellulose casings showed only moderate antilisterial activity in vacuum-sealed frankfurters, unless additional antimicrobials, such as potassium lactate and sodium diacetate, were employed (Luchansky and Call 2004). Nguyen et al. (2008) carried out similar experiments using an edible bacterial cellulose film containing nisin to control *L. monocytogenes* and total aerobic bacteria on the surface of vacuum-packaged frankfurters. The frankfurters packaged in films activated with 2,500 IU/ml showed significantly lower counts of *L. monocytogenes* and total aerobic plate counts during refrigerated storage for 14 days as compared to the controls. The authors concluded that activated cellulose films had potential applicability as antimicrobial packaging films or inserts for processed meat products. Another study reported that polythene films activated with

bacteriocin 32Y from *L. curvatus* were effective in reducing the population of listeria in vacuum-packaged frankfurters during storage at 4 °C (Ercolini et al. 2006). By using viable staining and fluorescence microscopy, the authors corroborated that the activated film caused an immediate reduction of live and appearance of dead cells just after 15 min from the packaging.

Another suggested application of nisin is the preservation of natural sausage casings. Casings derived from animal intestines can be one possible route for transmission of *C. perfringens* spores and other sulphite-reducing anaerobic spores, since the brining process of intestines does not inactivate bacterial endospores. In one study, it was shown that nisin was partly reversibly bound to casings and can reduce the outgrowth of *Clostridium sporogenes* spores in the model used by approximately 1 log cycle (Wijnker et al. 2011). This could open new possibilities to combat the entry of pathogens in the food chain.

In vacuum-packaged cooked ham, application of a gelatine coating gel containing a combination of lysozyme, nisin and EDTA in showed bactericidal activity for *B. thermosphacta*, *L. sakei*, *L. mesenteroides*, *L. monocytogenes* and *S. enterica* serovar Typhimurium (Gill and Holley 2000b). In sliced cooked ham packaged under MAP and stored at 4 °C, the inclusion of polyethylene/polyamide inserts coated with nisin (approximately 2,560 AU cm^2) reduced the levels of LAB, *Listeria innocua* and *Staphylococcus aureus*, and partially inhibited growth of total aerobic bacteria on the ham during storage (Scannell et al. 2000b). However, in ham steaks packaged in chitosan-coated plastic films containing 500 IU/cm^2 of nisin, the low bacteriocin concentration tested was ineffective in inhibiting *L. monocytogenes* (Ye et al. 2008).

Pediocin immobilization has also shown variable results. Encapsulation of pediocin AcH in liposomes enhanced its antimicrobial activity in meat slurries (Degnan and Luchansky 1992). However, in another study, when pediocin adsorbed to its heat-killed producer cells was used to treat sliced frankfurters before packaging, the number of *L. monocytogenes* decreased during 6 days of storage, but remained at constant levels for the remaining storage period (up to 21 days), indicating that the pediocin preparation was not efficient enough to kill all *L. monocytogenes* (Mattila et al. 2003). The efficacy of cellulose films containing pediocin PA-1/Ach (ALTA® 2351) against *L. innocua* and *Salmonella* sp. was tested on sliced ham packaged under vacuum and stored at 12 °C simulating abusive temperatures that can occur in supermarkets (Santiago-Silva et al. 2009). The antimicrobial films were more effective inhibiting growth of *L. innocua* (with a growth reduction of 2 log cycles compared to control treatment after 15 days of storage) than *Salmonella* (0.5 log cycle reduction in relation to control, after 12 days). However, the viable cell concentrations of the inoculated bacteria were not reduced for any of the treatments.

Films activated with enterocin 496K1 (from *Enterococcus casseliflavus* IM 416K1) and enterocins A and B have been tested in ready to eat meat products (Iseppi et al. 2008; Marcos et al. 2007). Enterocin 416K1 activated films reduced the levels of *L. monocytogenes* in contaminated frankfurters by ca. 1.5 to 0.5 log cycles within 24 h of storage at temperatures of 4 and 22 °C, but did not avoid expo-

nential growth of the pathogen during further storage of samples (Iseppi et al. 2008). Marcos et al. (2007) tested the antilisterial effects of enterocins A and B immobilized in different supports (alignate, zein and polyvinyl alcohol) on air-packed and vacuum-packed sliced cooked ham stored at 6 °C. The most effective treatment for controlling *L. monocytogenes* during storage was vacuum-packaging of ham with alginate films containing 2,000 AU/cm^2 of enterocins, with no increase from inoculated levels of *L. monocytogenes* until day 15.

Pre-surface application of bacteriocins in combination with post-packaging treatments is another approach of recent interest. Bacteriocin application followed by in-package thermal treatments can provide an effective combination to control *L. monocytogenes* on products such as frankfurters or turkey bologna, as shown for pediocin, nisin, nisin-lysozyme, or combinations of these bacteriocins with sodium lactate/sodium diacetate (Chen et al. 2004a; Mangalassary et al. 2008). Mangalassary et al. (2008) studied the efficacy of in-package pasteurization (65 °C for 32 s.) combined with pre-surface application of nisin and/or lysozyme to reduce and prevent the subsequent recovery and growth of *L. monocytogenes* during refrigerated storage on the surface of low-fat turkey bologna. In-package pasteurization in combination with nisin or nisin–lysozyme treatments was effective in reducing the population below detectable levels by 2–3 weeks of storage. In bologna manufactured with different sodium lactate/sodium diacetate combinations, dipping in pediocin solution followed by heat treatment decreased the *D*-values for inactivation of *L. monocytogenes* at low pediocin concentration, but exhibited a protective effect at higher concentrations, indicating that interactions between additives in formulations can vary at different temperatures/concentrations (Maks et al. 2010). In a previous study, treatments of frankfurters with 3,000 AU or 6,000 AU pediocin (in ALTA 2341) followed by heating in hot water reduced the populations of inoculated *Listeria* in proportion to the intensity of treatments (Chen et al. 2004a). The combination of pediocin (6,000 AU) with post-packaging thermal treatment (81 °C or more for at least 60 s), achieved a 50 % reduction of initial inoculation levels. Little or no growth of *L. monocytogenes* was observed on the treated frankfurters for 12 weeks at 4 or 10 °C, and for 12 days at 25 °C. This treatment did not affect the sensory qualities of frankfurters. The authors of this study concluded that pediocin (in ALTA 2341) in combination with postpackaging thermal treatment offers an effective treatment combination for improved control of *L. monocytogenes* on frankfurters.

Another example of a combined treatment is the application of nisin with pulsed light. Application of a Nisaplin dip followed by exposure to pulsed light (PL; 9.4 J/cm^2) reduced the population of *L. innocua* on sausages by 4 log cycles and inhibited its growth during refrigeration storage for 24–48 days (Uesugi and Moraru 2009). Since application of PL is approved for decontamination of food and food surfaces, the combined treatment could be applied as a post-processing step to reduce surface contamination and increase the safety of RTE meat products.

Bacteriocins have been proposed for use in packaged foods to increase the efficacy of irradiation treatments. One study reported that irradiation acted synergistically with pediocin on *L. monocytogenes* inoculated in packaged frankfurters (Chen et al. 2004b). Combination of pediocin with postpackaging irradiation at

1.2 kGy or more was necessary to achieve a 50 % reduction of *L. monocytogenes* on frankfurters. The combination of 6,000 AU of pediocin and irradiation at 2.3 kGy or more was the most effective treatment for inhibition of the pathogen for 12 weeks at 4 or 10 °C. Best results were reported on samples stored at 4 °C, with little or no growth of the pathogen during 12 weeks of storage and no adverse effects on the sensory quality of frankfurters. Similarly, bacteriocin-activated films have been tested as a way to increase the radiation sensitivity of the target pathogens, aimed at reducing radiation doses and impact on product quality. In ready-to-eat turkey meat vacuum-packaged with a pectin-nisin film and treated by low dose irradiation (2 kGy), the reduction obtained for the *L. monocytogenes* population (5.36 log CFU/cm^2) were greater compared to irradiation and pectin film single treatments. In addition, pectin-nisin films did significantly slow the proliferation of *L. monocytogenes* cells that survived irradiation during 8 weeks of storage at 10 °C (Jin et al. 2009). The authors concluded that the combined treatment could serve to prevent listeriosis due to postprocessing contamination while reducing radiation doses and impact on product quality, or to prevent *L. monocytogenes* growth in accidentally recontaminated packages of irradiated RTE meats.

High hydrostatic pressure processing (HHP) is now being used more frequently as a food processing technology that is applied on packaged foods. Several reports indicate that bacteriocins can enhance the antibacterial effects of HHP treatments. In one study, the efficacy of enterocins added to cooked ham increased in combination with a HHP treatment at 400 MPa for 10 min (Garriga et al. 2002). The combined treatment avoided overgrowth of *L. sakei* CTC746 strain during storage, improving the results compared to HHP treatment alone (Garriga et al. 2002). *L. monocytogenes* was also kept at levels <10 CFU/g for 61 days at 4 °C (Garriga et al. 2002). However, the bacteriocins had no effect on regrowth of other survivors (*Ln. carnosum* CTC747, *Staphylococcus carnosus* and *S. aureus* strains, *E. coli* or *S. enterica* strains). A combined treatment of enterocins (2,400 AU/g) and HHP (400 MPa, 10 min) avoided overgrowth of surviving listeria upon a simulated cold-chain break event when the samples were stored at 1 °C, but not at 6 °C (Marcos et al. 2008a), indicating the influence of storage temperature on the delicate balance between inhibited proliferation of survivors and repair of sublethal damage and cell growth.

Protective coatings in the form of activated films have also been tested to increase the efficacy of HHP in ready-to-eat meat products (Aymerich et al. 2008). The efficacy immobilized enterocins in combination with HHP to control *L. monocytogenes* growth during the shelf life of artificially inoculated cooked ham was investigated (Jofré et al. 2007). The antilisterial activity of enterocins immobilised in plastic interleaves was strongly potentiated by application of HHP treatment (400 MPa, 10 min), reducing viable counts by about 4 log units and holding the levels of *L. monocytogenes* in the treated sliced ham below 1.5 log CFU/g at the end of storage for 30 days at 6 °C (Jofré et al. 2007). Storage of samples at a lower temperature of 1 °C extended the protective effect of the combined treatment for at least 60 °C, even in the event of a simulated cold chain break (Marcos et al. 2008b). In a separate study (Marcos et al. 2008a), sliced cooked ham was packaged in alginate films containing or not enterocins A and B, and then was pressurized (400 MPa, 10 min, 17 °C).

While the single antimicrobial packaging treatment was able to inhibit growth of *L. monocytogenes* during the first 8 days of storage at 6 °C, and the single HHP pretreatment attained a ca. 3.4 logs reduction of viable counts for about the same period followed by regrowth of the listeria in both cases, the combined treatment extended the lag phase of listeria to 22 days, and the slight growth observed afterwards did not exceed 1.8 log CFU/g by the end of storage (day 60). For samples stored at 1 °C, the combined treatment of HHP and enterocin film caused a faster decline of *L. monocytogenes* counts compared to HHP alone, but no regrowth was observed in either case for 60 days, suggesting that at the lower temperature of storage, antimicrobial packaging did not give additional protection against *L. monocytogenes* to pressurized samples. However, after a simulated cold-chain break event at day 60, there was a dramatic increase in the *L. monocytogenes* population for single HHP treatments (8.5 log CFU/g), indicating the capacity of pressure-injured *L. monocytogenes* cells to recover under favourable conditions. By contrast, for the combined treatment of HHP and enterocin films, the temperature abuse resulted in a slight increase until 1.7 log CFU/g at 90 days. The authors concluded the combination of antimicrobial packaging with HPP could be useful to control and reduce the numbers of *L. monocytogenes* and to overcome temperature abuse. In a similar study, Jofré et al. (2008) tested the effectiveness of the application of interleavers (composed by polypropylene/polyamide layers) containing enterocins A and B, sakacin K, nisin A, potassium lactate and nisin plus lactate alone or in combination with a 400 MPa HHP treatment in sliced cooked ham spiked with *Salmonella* spp. It was concluded that nisin was the only treatment that produced absence of *Salmonella* 24 h after pressurisation and the application of nisin through interleavers and combined with an HHP treatment appears as the most effective treatment to achieve absence of *Salmonella* in 25 g samples during refrigeration storage of the sliced ham (Jofré et al. 2008).

4.1.3 Fermented Meats

Bacteriocin preparations can be added to meat batters for reduction of the initial levels of bacteriocin-sensitive populations and inactivation of microbial pathogens in fermented meat products. The lower pH attained in sausages compared to fresh meats may increase the solubility of some bacteriocins like nisin, and probably their antimicrobial activity as well. Microbial inactivation by bacteriocin addition may also be an attractive hurdle for slightly fermented sausages, in which the higher pH and water content may facilitate survival and proliferation of certain pathogenic bacteria.

Several bacteriocins such as nisin, enterocins (CCM 4231, A, B and AS-48) or leucocins improved the reduction of *L. monocytogenes* or *S. aureus* populations in fermented meats (Rodríguez et al. 2002; Chen and Hoover 2003; Aymerich et al. 2008; Galvez et al. 2008). Addition of nisin alone was effective in preservation of bologna-type sausages against LAB spoilage (Davies and Delves-Broughton 1999) and in the inhibition of *L. monocytogenes* in sucuk, a Turkish fermented sausage

(Hampikyan and Ugur 2007). The effectiveness of nisin in fermented meats increased in combination with other antimicrobials, such as organic acids (reducing the viable counts of *S.* Kentucky and *S. aureus*; Scannell et al. 1997), lysozyme-EDTA (inhibiting the growth of *B. thermosphacta*, *L. curvatus*, *Ln. mesenteroides*, *L. monocytogenes* and *E. coli* O157:H7; Gill and Holley 2000a) or grape seed extract (Sivarooban et al. 2007). Enterocins can inhibit *Listeria* in fermented meats, as shown for enterocin CCM 4231 in dry fermented Hornád salami (Lauková et al. 1999) or enterocins A and B in espetec (traditional Spanish sausage; Aymerich et al. 2000). Addition of enterocin CCM 4231 (12,800 AU/g) from *E. faecium* CCM 4231 to Hornád salami meat mixture resulted in a reduction of *L. monocytogenes* by 1.67 log cycle immediately after addition of the bacteriocin (Lauková et al. 1999). Although the added bacteriocin did not prevent growth of the listeria during storage of samples in drying rooms at temperatures between 24 and 15 °C, viable counts were significantly lower that the controls. In espetec (a Spanish slightly-fermented sausage), addition of enterocins A and B (648 AU/g) reduced the viable counts of *L. innocua* below 50 CFU/g from the fifth day until the end of the process (12 days) of manufacturing (Aymerich et al. 2000).

In Italian sausages ("cacciatore"), enterocin 416K1 (10 AU/g, in the form of a concentrated culture supernatant) decreased the levels of *L. monocytogenes* in sausages by ca. 2.5 log CFU/g during the drying period (3 days), but failed to suppress the pathogen during ripening (Sabia et al. 2003). Regarding enterocin AS-48, after addition of this bacteriocin at 450 AU/g in a meat sausage model system, it was observed that no viable listeria were detected after 6 and 9 days of incubation at 20 °C (Ananou et al. 2005a), and also that viable counts of *S. aureus* were reduced below detectable levels at the end of storage (Ananou et al. 2005b). Also bacteriocins from leuconostocs have been tested in fermented meats. Addition of semi-purified bacteriocin of *Ln. mesenteroides* E131 improved the reduction of *L. monocytogenes* viable counts in challenge experiments during fermented sausage manufacturing (Drosinos et al. 2006).

4.2 Application of Protective Cultures

4.2.1 Raw Meats

Many LAB naturally associated with meats can grow at refrigeration temperatures. Therefore, bacteriocin-producing strains of these LAB that do not have adverse effects on meats can be selected as protective cultures for raw meat preservation (Table 4.2). Previous works have demonstrated the effectiveness of bacteriocin-producing *L. sakei* and *L. curvatus* strains in inhibiting *L. monocytogenes* or *B. thermosphacta* in raw meat products. When *L. sakei* CWBI-B1365 and *L. curvatus* CWBI-B28 (producers of sakacin G and P, respectively) were tested as protective cultures on raw beef and poultry meat challenged with *L. monocytogenes* and stored at 5 °C in sealed bags, inhibition of the listeria was found to depend greatly on the

Table 4.2 Examples of applications of bacteriocin-producing cultures in meat and poultry products

Starter or protective cultures		
Raw meats		
Bacteriocin producer *L. curvatus* CRL705	Effective inhibition of *L. innocua* and *B. thermosphacta* and indigenous contaminant LAB in fresh beef; contribution to meat ageing by limited proteolysis	Fadda et al. 2008
BLIS-producing *L. sakei*	Delayed blownpack spoilage caused by *C. estertheticum* and reduced survival of *C. jejuni* on meat	Jones et al. 2009
BLIS-producing *L. fermentum* ACA-DC179	Growth inhibition of *S. Enteritidis* in refrigerated chicken ground meat	Maragkoudakis et al. 2009
RTE meats		
Bacteriocin-producing *P. acidilactici* strains	Inhibition of *L. monocytogenes* in cooked meats	Rodríguez et al. 2002
Sakacin K-producing *L. sakei* CTC494	Inhibition of *L. monocytogenes* in cooked meat products	Hugas et al. 1998
Bacteriocin-producing *L. sakei*	Growth inhibition of *L. monocytogenes* and *E. coli* O157.H7 in cooked, sliced, vacuum-packaged meats	Bredholt et al. 1999
Fermented meats		
Bacteriocin-producing *L. sakei* starter cultures	Reduction of *Listeria* populations in fermented sausages	Ravyts et al. 2008
Curvacin-producing *L. curvatus*	Antilisterial effects in meat fermentation	Dicks et al. 2004
Pediocin-producing *P. acidilactici*	Commercial starter cultures for fermentation of meat products to reduce the numbers of *L. monocytogenes* in the final product	Amezquita and Brashears 2002
E. faecalis CECT7121 (producer of enterocin MR99)	Reduction of viable counts of *Enterobacteriaceae*, *S. aureus* and other Gram-positive cocci in craft dry-fermented sausages	Sparo et al. 2008

meat substrate (Dortu et al. 2008). On raw beef, *L. curvatus* CWBI-B28 was more effective in reducing *L. monocytogenes* cell concentrations below detectable levels (7 days) than *L. sakei* CWBI-B1365 (21 days). In poultry meat, the application of the LAB strains separately showed much lower inhibitory activities, but their addition in combination led to growth inhibition of the listeria. This is an interesting example of a synergistic effect between two sakacin-producing strains in a food system.

Lactobacillus curvatus CRL705 used as a protective culture in fresh beef was effective in inhibiting *L. innocua* and *B. thermosphacta* as well as the indigenous contaminant LAB at low temperatures and had a negligible effect on meat pH (Castellano et al. 2008). It was observed that meat inoculation with *L. curvatus* CRL705 showed a net increase of free amino acids, due to the complementary activity of the bacterial and meat proteases on meat sarcoplasmic proteins (Fadda et al. 2008).

It was proposed that *L. curvatus* CRL705 protective cultures could contribute to meat ageing by generating small peptides and free amino acids, while improving shelf life (Fadda et al. 2008).

Inoculation with a sakacin A producer *L. sakei* strain reduced the population of *L. monocytogenes* on vacuum-packed lamb during 12 week storage. Similarly, inoculation with BLIS-producing *L. sakei* strains delayed blownpack spoilage caused by *Clostridium estertheticum* and reduced the survival of *Campylobacter jejuni* on beef meat (Jones et al. 2009). In vacuum-packaged chicken cuts, inoculation with sakacin-P producing *L. sakei* achieved a growth inhibition of *L. monocytogenes* (Katla et al. 2002). Plantaricin-producing *L. plantarum* showed anti-listerial effects in uncooked and cooked chicken meat (Enan 2006; Gamal 2006). Enterococci have also been tested as protective cultures in raw meats. In chicken ground meat stored at 8–10 °C, growth of *L. monocytogenes* and *S*. Enteritidis was adversely affected by the respective presence of protective cultures consisting of strain *E. faecium* PCD71 (carrying the genetic determinants for enterocins A, P, L50A and L50B) and strain *L. fermentum* ACA-DC179, producer of BLIS against *Salmonella* (Maragkoudakis et al. 2009; Zoumpopoulou et al. 2008). Strain *E. faecium* PCD71 inhibited the growth of *L. monocytogenes* by at least 0. 7 log CFU/g after 7 days storage, while strain *L. fermentum* ACA-DC179 inhibited the growth of *S*. Enteritidis by up to 1.3 log CFU/g compared to the control (Maragkoudakis et al. 2009). In addition, none of these two strains caused detrimental effects on biochemical parameters related to spoilage of the chicken meat.

4.2.2 Semi-processed and Cooked Meats

Lactic acid bacteria are the prevalent spoilage microorganisms in cooked meat products (Mataragas et al. 2006, Audenaert et al. 2010, Chenoll et al. 2007). The shelf life of most heat processed meats is limited by *Lactobacillus* and *Leuconostoc* strains that rapidly recontaminate the product during handling and slicing (Lücke 2000). These LAB also tend to displace pathogenic bacteria. In the absence of competing microbiota, *L. monocytogenes* will proliferate more easily. Specific bacteriocin-producing LAB strains could be used as protective cultures for semi-processed and cooked meats provided that they cause only a minimal change in the desired sensory properties of the products while inhibiting *Listeria* and displacing other LAB involved in spoilage (Hugas et al. 1998; Lücke 2000; Chen and Hoover 2003; Aymerich et al. 2008; Galvez et al. 2008). Bacteriocin-producing protective cultures have been shown to inhibit *L. monocytogenes* in vacuum-packaged processed meats, such as *Lactobacillus bavaricus* MN in minimally heat-treated beef cubes (Winkowski et al. 1993), *P. acidilactici* JBL 1095 in wieners (Degnan et al. 1992), or *P. acidilactici* JD1-23 in frankfurters (Berry et al. 1991). In Brazillian raw sausage lingüiça, bacteriocin-producing *Lactobacillus sake* 2a also inhibited growth of *L. monocytogenes* (Liserre et al. 2002). The bacteriocinogenic strains *L. sakei* CTC494

and *E. faecium* CTC492 (producer of enterocins A and B) prevented slime formation in cooked pork by *Lb. sakei* but not by *Leuconostoc mesenteroides* (Aymerich et al. 1998). In sliced, vacuum-packaged cooked ham, the same enterococcal strain partially prevented ropiness by *L.sakei* (Aymerich et al. 2002). Inoculation of strains producing sakacin P or leucocin in cooked meat products was shown to inhibit growth of listeria (Katla et al. 2002; Jacobsen et al. 2003), and protective *L. sakei* cultures were also shown to inhibit *L. monocytogenes* and *E. coli* O157:H7 in vacuum-packed cooked meat products (Bredholt et al. 1999). The bacteriocinogenic strain *L. curvatus* CWBI-B28 reduced *L. monocytogenes* levels below detection limits in bacon meat within 1 or 2 weeks in absence or presence of nitrites, respectively (Ghalfi et al. 2006). Anti-listerial effect was also observed with a plantaricin producing *L. plantarum* strain in cooked chicken meat (Enan 2006). There are already several LAB cultures in the market introduced as starter or bioprotective culture with the aim of contributing to microbiological safety of semi-processed and cooked meats (Aymerich et al. 2008).

4.2.3 Fermented Meats

Certain lactic acid bacteria play key roles in meat fermentations. Therefore, bacteriocin-producing strains have been proposed as starter cultures to combat pathogens such as *L. monocytogenes* (Työppönen et al. 2003; Leroy et al. 2006; Aymerich et al. 2008). Bacteriocin-producing lactobacilli (mainly *L. sakei* and *L. curvatus*, but also *Lactobacillus rhamnosus* and *L. plantarum*) have demonstrated anti-listerial effects in sausage or salami fermentations, depending to a great extent on strain and type of meat (Erkkilä et al. 2001; Leroy et al. 2005; Dicks et al. 2004; Benkerroum et al. 2005; Todorov et al. 2007) (Table 4.2).

L. sakei CTC 494 (producing sakacin K) is a promising functional starter culture with antilisterial activity, being capable to successfully suppress *L. monocytogenes* in Spanish-style and German-style fermented sausages (Aymerich et al. 2008) or to reduce listeria populations in Belgian-style sausages, Italian salami, and Cacciatore salami (Ravyts et al. 2008). The efficacy of *L. sakei* is influenced by environmental factors such as sausage ingredients, salt, fat and nitrite content, acidification level, and temperature (Leroy et al. 2006). Since *L. sakei* and *L. curvatus* can hydrolyze muscle sarcoplasmic proteins and, in a lesser extent, myofibrillar proteins, they can contribute to the generation of small peptides and amino acids which contribute as direct flavour enhancers or as precursors of other flavour compounds during the ripening of dry-fermented sausages (Leroy et al. 2006). Exploitation of these activities may lead to the use of a new generation starter cultures with industrial or nutritional important functionalities (Leroy et al. 2006). Another, yet unexplored possible application of these functional properties would be the generation of bioactive peptides from the meat proteins by selected LAB with adequare proteolytic activities.

Bacteriocin-producing pediococci can reduce *L. monocytogenes* populations in fermented meats (Amezquita and Brashears 2002; Rodríguez et al. 2002; Aymerich

et al. 2008). Pediococci are preferred as starters in certain products (rather than lactobacilli), e.g. in American-style sausages fermented at higher temperatures. Bacteriocin-producing pediococci were proposed as indigenous starter cultures in the fermentation of Urutan, a Balinese traditional dry fermented sausage (Antara et al. 2004). One advantage is that pediocin PA-1 producers do not inhibit bacteria relevant to the fermentation such as staphylococci and micrococci (Gonzalez and Kunka 1987).

Enterococci are often part of the normal microbiota in meat fermentations, and have demonstrated to be effective as antilisteria agents in fermented meats, being also able to inhibit *S. aureus* (Foulquié Moreno et al. 2003; Aymerich et al. 2008; Galvez et al. 2008). However, their application in foods is controversial because of their potential virulence as opportunistic pathogens and also as carriers of antimicrobial resistance genes. The bacteriocinogenic strains *E. faecium* CCM 4231 and *E. faecium* RZS C13 strongly inhibited the growth of *Listeria* spp. in sausage fermentations (Callewaert et al. 2000), and *Enterococcus casseliflavus* IM 416K1 (producer of enterocin 416K1) was able to suppress *L. monocytogenes* in artificially inoculated "cacciatore" Italian sausages (Sabia et al. 2003). During sausage fermentation, inoculated *Enterococcus faecalis* A-48-32 (producer of the broad-spectrum cyclic enterocin AS-48) or its transconjungant *E. faecium* S-32-81, reduced the concentration of *L. monocytogenes* down to undetactable levels within 7 or 6 days of incubation at 20 °C (Ananou et al. 2005a). Similarly, strain A-48-32 inhibited growth of *S. aureus* and reduced viable cell counts to 1 log CFU/g at the end of fermentation (Ananou et al. 2005b). Strain *E. faecalis* CECT 7121 (isolated from natural corn silage, and producer of the broad-spectrum enterocin MR99) is interesting because it is devoid of the genes for haemolysin and gelatinase production, and does not produce biogenic amines (Sparo et al. 2008). When tested in the manufacture of craft dry-fermented sausages, the sausages inoculated with *E. faecalis* CECT 7121 had lower viable counts of *Enterobacteriaceae*, *S. aureus* and other Gram-positive cocci at the end of fermentation (2 days), with no detectable enterobacteria and *S. aureus* at the end of drying (21 days). *E. faecalis* CECT7121 did not affect the growth of *Lactobacillus* spp. but it displaced the autochthonous populations of enterococci (Sparo et al. 2008).

The potential of bacteriocin-producing lactococci in meat fermentations has been studied to a much less extent. Nisin-producing lactococcal strains isolated from fermented sausages were suggested as adjunct cultures for improving the food safety of meat fermented products manufactured under poor hygienic conditions such as indigenous fermentations (Rodriguez et al. 1995; Noonpakdee et al. 2003). Furthermore, it was reported that a transformant *L. lactis* strain producing lacticin 3417 significantly reduced the populations of *L. innocua* and *S. aureus* in sausages, although growth of the bacteriocin producer was markedly influenced by sausage ingredients (Scannell et al. 2001). In another study on manufacture of merguez, a dry-fermented beef meat sausage, inoculation with the Bac+ strain *L. lactis* subsp. *lactis* M significantly reduced the levels of *L. monocytogenes* during the fermentation phase (Benkerroum et al. 2003). However, inoculation with a lyophilized culture of the bacteriocin-producing strain *L. lactis* LMG21206 decreased *Listeria* counts to

below the detectable limit after 15 days of drying, but it had no effect on the viability of the listeria during sausage fermentation. By comparison, the results obtained with the Bac + strain *L. curvatus* LBPE were superior, with highly significant reductions during fermentation and ripening (Benkerroum et al. 2005).

Several LAB strains may antagonise growth o *E. coli* O157:H7 in fermented sausages. This inhibitory effect has been attributed to the production of small antimicrobial compounds (such as reuterin, 3-hydroxy fatty acids, phenyllactic acid, and 4-hydroxyphenyllactic acid and novel bacteriocins; Leroy et al. 2006). It was shown that inoculation of salami with strains of *Lactobacillus* spp. as well as bifidobacteria reduced the levels of *L. monocytogenes* and *E. coli* O111 during fermentation of sausage batter (Pidcock et al. 2002). Similar results were reported for *Lactobacillus reuteri* and *Bifidobacterium longum* in dry fermented sausages. In the treatment containing *L. reuteri* (producer of reuterin), a 3 log CFU/g reduction in *E. coli* O157:H7 numbers was found at the end of drying, while *B. longun* was reported to have lower effects (1.9 log CFU/g reduction) (Muthukumarasamy and Holley 2007).

Staphylococci and micrococci may also be exploited as sources for antibacterial substances applicable in sausage fermentations. The introduction of the lysostaphin gene (an endopeptidase that specifically cleaves the glycine–glycine bonds unique to the interpeptide cross-bridge of the *S. aureus* cell wall) into meat starter lactobacilli (Cavadini et al. 1998) is an interesting approach to prevent the growth of *S. aureus*. Furthermore, one *Staphylococcus xylosus* sausage isolate that produces an antilisterial substance increased the microbial inactivation of *L. monocytogenes* in Naples-type sausage (Villani et al. 1997). *Kocuria varians* (formerly *Micrococcus varians*) produces the lantibiotic variacin (Pridmore et al. 1996). Strains producing this lantibiotic were isolated form Italian-type raw salami fermentations. Bacteriocinogenic *Kocuria* strains could be very interesting as adjunct protective cultures in meat fermentations.

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Chapter 5
Biopreservation of Milk and Dairy Products

5.1 Application of Bacteriocin Preparations

5.1.1 Raw Milks

Milk may act as vehicle for human pathogenic bacteria (reviewed by Claeys et al. 2013). Pasteurization of milk before human consumption or for the manufacture of dairy products is often required or recommended. Pasteurizarion will decrease the background spoilage microbiota, but it will not yield a sterile product. Some traditional, highly appreciated fermented dairy foods are still made from raw milk, and there is an ongoing debate on the benefits of consuming raw milk versus pasteurized milk (Claeys et al. 2013). According to foodborne disease reports from different industrialized countries, milk and milk products are implicated in 1–5 % of the total bacterial foodborne outbreaks, with 39.1 % attributed to milk, 53.1 % to cheese and 7.8 % to other milk products (De Buyser et al. 2001; Claeys et al. 2013). Bacteriocins seem an attractive approach to improve the safety of milk and dairy products (especially in those made from raw milk), and at the same time may offer some potential technological applications such as in acceleration of cheese ripening (Table 5.1). The antimicrobial effects of bacteriocins and/or their produced strains have been investigated both in raw milks and in several types of dairy products.

Many different bacteriocins preparations have been tested for preservation of milks and dairy products, with the purpose of inactivating foodborne pathogenic or spoilage bacteria. Addition of nisin to raw milks may help solving particular shelf-life problems associated with hot weather temperature and/or long distance transport and inadequate refrigeration systems (Davies and Delves-Broughton 1999; Thomas et al. 2000). The application of nisin in combination with heat treatments decreased the D values of bacteria such as *Bacillus cereus* and *Geobacillus stearothermophilus* and natural microbiota, making it possible to apply milder thermal treatments and at the same time extend the shelf life of milk even under poor refrigeration conditions. Another suggested approach was to use coatings containing

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Table 5.1 Examples of bacteriocin applications in dairy foods

Bacteriocin treatment	Effect(s)	Reference(s)
Nisin	Prevent proliferation of surviving endospore formers, mainly the gas-producing clostridia and *C. botulinum* in cheeses	Thomas and Delves-Broughton 2001
	Prevent post-process contamination with *L. monocytogenes*	
Nisin and PEF	Increased antimicrobial activity in milks against several bacteria such as *L. monocytogenes, S. aureus, B. cereus* and *E. coli*	Sobrino-López and Martín-Belloso 2008
Nisin and HHP	Increased the inactivation of spoilage bacteria associated with milk	Black et al. 2005
Lacticin 3147	Inactivation of *L. monocytogenes* in reconstituted demineralized whey poder and *S. aureus* in reconstituted skimmed milk	Morgan et al. 2000
Lacticin 3147	Inactivation of *L. monocytogenes* in natural yogurt and in Cottage cheese	Morgan et al. 2001
Enterocin AS-48	Rapid inactivation of *L. monocytogenes* and slower inhibition of *S. aureus* in skim milk	Ananou et al. 2010
Enterocin AS-48 and PEF	Enhanced inactivation of *S. aureus* in skim milk	Sobrino et al. 2009
Pediocin PA-1/AcH	Inhibition of *L. monocytogenes* in several dairy systems (dressed Cottage cheese, half-and-half cream, cheese sauce, and others)	Rodríguez et al. 2002
Lacticin 3147-producing cultures	Inhibition *L. monocytogenes* in Cottage cheese, and on the surface of a mould-ripened cheese and a smear-ripened cheese	O'Sullivan et al. 2006
Enterocin AS-48 producer *E. faecalis* strain	Inhibition of *B. cereus* and *S. aureus* in cheeses and in skim milk	Muñoz et al. 2004, 2007
Lacticin 3147-producing *L. lactis* IFPL 3593	Inhibition of gas formation by *C. tyrobutyricum* and heterofermentative lactobacilli in cheese	Martínez-Cuesta et al. 2010
Bacteriocin producer *L. gasseri* K7	Reduced outgrowth of inoculated *C. tyrobutyricum* and butyric acid formation in the cheeses. Probiotic properties	Bogovič Matijašić et al. 2007
S. macedonicus ACA-DC (producer of macedocin)	Inhibition of gas formation by *C. tyrobutyricum* in cheese	Anastasiou et al. 2009
Lacticin 481-producing culture	Prevention of late-blowing defects due to inoculated *C. beijerinckii*	Garde et al. 2011
Lacticin 3147-producing cultures	Increased generation of 2-methylbutanal with the concomitant enhancement of the cheese aroma	Fernández de Palencia et al. 2004
Lacticin 3147-producing cultures	Inhibition of adventitious non-starter LAB flora during ripening, enhancing the cheese quality	Ryan et al. 2001

immobilized nisin for milk packaging. In experimental trials, a low-density polyethylene film coated with nisin retarded growth of *Micrococcus luteus* as an indicator strain in raw, pasteurized and UHT milk (Mauriello et al. 2005). Similar results were reported with virgin paperboard coated with nisin and/or chitosan in a binder of vinyl acetate–ethylene copolymer during storage of pasteurized milk (Lee et al. 2004), or with cross-linked hydroxypropylmethylcellulose (HPMC) films containing nisin (Sebti et al. 2003).

The antibacterial activity of nisin in milk can be enhanced in combination with other antimicrobials, such as monolaurin, the lactoperoxidase (LPS) system, lysozyme or reuterin (Gálvez et al. 2007), or even by addition of bioactive culture supernatants as reported for *Bacillus licheniformis* ZJU12 (He and Chen 2006). Application of nisin in combination with pulsed-electric fields (PEF) or high hydrostatic pressure (HHP) has been investigated in recent years with the purpose of increasing the efficacy of treatments while having lower impact on the food organoleptic properties and nutritional value (Black et al. 2005; Sobrino-López and Martín-Belloso 2008). The combined application of PEF and nisin was shown to improvethe inactivation of *Listeria monocytogenes, Staphylococcus aureus, B. cereus* and *Escherichia coli* in pumpable substrates such as skim milk, whey, or simulated milk ultrafiltrate media (Calderón-Miranda et al. 1999; Pol et al. 2001; Terebiznik et al. 2002; Sobrino-López et al. 2006; Sobrino-López and Martín-Belloso 2008). Increasing the electric field intensity, the number of pulses and the nisin concentration acted synergistically for inactivation of *Listeria innocua* in skim milk, achieving up to 3.8 log units inactivation after application of a PEF treatment of 32 pulses at 50 kV/cm in combination with 100 IU/ml nisin (Calderón-Miranda et al. 1999). Similar reductions of cell viability were observed on the natural microbiota of raw milk (Smith et al. 2002) and on *S. aureus* inoculated in skim milk (Sobrino-López et al. 2006). Synergy between PEF treatment and nisin may be further enhanced by inclusion of a third hurdle, such as a mild thermal treatment or the addition of other antimicrobials such as carvacrol or lysozyme (Sobrino-López and Martín-Belloso 2008). For example, the combination of PEF treatment (50 pulses at 80 kV/cm and 52 °C) with nisin (38 IU/ml) and lysozyme (1,638 IU/ml) achieved at least 7.0 log reduction of the milk endogenous microbiota (Smith et al. 2002).

Application of combined treatments of nisin and HHP (with or without lysozyme) increased the inactivation of bacteria associated with milk such as *E. coli, Pseudomonas fluorescens, L. innocua*, and *Lactobacillus viridescens* (García-Graells et al. 1999; Black et al. 2005). For example, treatment at 500 MPa for 5 min in combination with nisin (500 IU/ml) completely inactivated *P. fluorescens* and *E. coli* and reduced *L. innocua* by more than 8.3 log cycles, while reductions of cell viability obtained for the single treatments were considerably lower, in the range of 1.5–3.8 log cycles (Black et al. 2005). Milk exerts a protective effect on bacteria against HHP. For that reason, and also to overcome the higher resistance of some bacteria, nisin was combined with other antimicrobials in HHP treatments. For example, the combination of nisin and pediocin PA-1 (500 AU/ml total activity) with HHP treatment (345 MPa, 50 °C, 5 min) reduced viable cell counts of *S. aureus* by more than 8-log cycles, and also avoided growth of possible survivors for at least

30 days of storage (25 °C) of the treated milk samples (Alpas and Bozoglu 2000). In another study, the combination of nisin and lysozyme with HHP improved inactivation of pressure-resistant *E. coli* in skim milk, although the efficacy of the combined treatment decreased as the fat content of milk increased (García-Graells et al. 1999).

Lacticin 3147 is another lactococcal bacteriocin with a high potential for application in the preservation of dairy foods (Ross et al. 1999; O'Sullivan et al. 2002b). The effects of lacticin 3147 preparations on the inactivation of *L. monocytogenes* in reconstituted demineralized whey poder and *S. aureus* in reconstituted skimmed milk. Lacticin addition at 10,000 AU ml achieved a 0.7 log reduction in viability for both bacteria after 30 min incubation, while 20,000 AU achieved a 2.1 log reduction of the listeria population (Morgan et al. 2000). Microbial inactivation increased greatly when lacticin preparations were tested in combination with HHP treatments. The combination of 250 MPa and lacticin 3147 resulted in more than 6 logs of kill for both bacteria. It was also shown that the antibacterial activity in concentrated preparations of this lacticin against *L. monocytogenes* and *S. aureus* could be enhanced in combination with HHP treatment (150–275 MPa), thereby reducing the amounts of bacteriocin required (Morgan et al. 2000).

Only a few enterocins have been investigated in milks. For example, enterocin CCM 4231 was able to inhibit *L. monocytogenes* and *S. aureus* in milk (Lauková et al. 1999). Enterocin AS-48 (50 μg/ml) added to skim cow milk partially reduced the population of inoculated *S. aureus* during the first 10 h of incubation at 28 °C, but did not avoid overgrowth at 24 h (Muñoz et al. 2007). However, a lower bacteriocin dose (20 μg/ml) in combination with a mild heat treatment (65 °C, 5 min) was highly effective in reducing the population of staphylococci in the milk below detectable levels within the first 8 h of incubation and also to avoid overgrowth of staphylococci at 24 h. *Enterococcus faecalis* INIA 4 produces enterocin 4, which is identical to enterocin AS-48. In bacteriocin filtrates prepared by cultivation of this strain on skimmed ewe's milk, the levels of inoculated *L. monocytogenes* Ohio and Scott A strains were reduced by 3.23 and 2.13 log CFU/ml respectively, after 24 h incubation at 30 °C. Reductions obtained under similar conditions for a collection of *L. monocytogenes* strains isolated from dairy environments were in the range of 0.52–3.48 log cycles (Rodríguez et al. 1997). Enterocin AS-48 can now be produced as a dry powder based on a whey permeate that is essentially free of dairy allergens. Addition of this bacteriocin preparation to skim milk, rapidly inactivated the inoculated *L. monocytogenes* cells and progressively reduced the counts of *S. aureus* (Ananou et al. 2010).

Microbial inactivation in skim milk improved when bacteriocins were tested in combination with PEF. The combinations of enterocin AS-48 with or without nisin and PEF treatment increased the inactivation of *S. aureus* (Sobrino et al. 2009). A maximum of 4.5 logs reduction was achieved for AS-48 (28 AU/ml) and PEF (35 kV/cm). This reduction factor increased up to 6 logs when 28 AU/ml of AS-48 and 20 IU/ml of nisin were added to the milk before PEF treatment. The combined treatment extended the shelf life of milk by at least 1 week compared to a conventional pasteurisation treatment.

5.1.2 Processed Milk Products

Nisin is widely used in the dairy industry for inhibition of gas blowing defect in cheeses caused by *Clostridium tyrobutyricum*, but also in processed cheeses and cheese products to inhibit *Clostridium botulinum*, and to prevent growth of post-process contaminating bacteria such as *L. monocytogenes* (Davies and Delves-Broughton 1999; Thomas et al. 2000; Thomas and Delves-Broughton 2001; Deegan et al. 2006; Sobrino-López and Martín-Belloso 2008) (Table 5.1). It is also used in many other pasteurised dairy products, such as chilled desserts, flavoured milk, clotted cream, or canned evaporated milks (Thomas et al. 2000). For example, the addition of nisin powder to milk in the production of cheese made without a starter culture can control microbial contamination (Sobrino-López and Martín-Belloso 2008). Addition of nisin at 100 or 500 mg/kg suppressed total plate and anaerobic spore counts in processed cheese during 3 months of storage at 5 or 21 °C, and even the growth of *G. stearothermophilus*, *B. cereus* and *Bacillus subtilis* were inhibited by 5 mg/kg nisin (Plockova et al. 1996). In Ricotta-type cheese, addition of nisin (2.5 mg/l) inhibited the growth of *L. monocytogenes* for more than 8 weeks, while cheese made without nisin contained unsafe levels of the bacteria within 1–2 weeks. In addition, there was a high level of retention of nisin activity in the cheese after 10 weeks of incubation at 6–8 °C, with only 10–32 % loss of antibacterial activity (Davies et al. 1997).

Nisin addition in combination with HHP could be useful for inactivation of endospores and mesophilic bacteria in cheese (Capellas et al. 2000; López-Pedemonte et al. 2003; Arqués et al. 2005a). For example, the combination of nisin with HHP strongly reduced the counts of *B. cereus* spores in a traditional cheese curd. Since bacterial endospores are pressure-resistant, two HHP cycles were applied, the first one to induce endospore germination and the second one to destroy the vegetative cells (López-Pedemonte et al. 2003). The authors of this study concluded that the combined treatment could improve the microbial stability and safety of cheeses, especially those made from unpasteurised milk, such as many traditional cheeses, decreasing the restrictions that are currently imposed on the commercialisation of such cheeses.

Due to its non-specific inhibitory activity against Gram-positive bacteria, nisin may interfere with growth of starter cultures cheese fermentation and have detrimental effects on acidification and/or aroma formation. In order to solve this limitation and also to enhance nisin stability, nisin Z was encapsulated in liposomes (Benech et al. 2002a, b). Addition of liposome preparations was shown to inhibit listeria in Cheddar cheese (Benech et al. 2002a, b). In another study, nisin encapsulated in soybean phosphatidylcholine nanovesicles provided best results compared to added free nisin in the control of *L.monocytogenes* in Minas frescal cheese stored at 7 °C, keeping the concentrations of listeriae lower than the untreated controls for at least 21 days (Malheiros et al. 2012). Some growth inhibition was also obtained with an encapsulated BLIS P34 (derived from *Bacillus* sp. P34) in parallel experiments.

The authors of this study concluded that encapsulation of bacteriocins in liposomes of partially purified soybean phosphatidylcholine may be a promising technology for the control of foodborne pathogens in cheeses.

Processing of dairy foods, such as cheese slicing, can be a critical point for bacterial contamination. For application on sliced cheeses, nisin immobilized in polyethylene/polyamide packagings was shown to reduce the population of LAB, *L. innocua* and *S. aureus* on the cheese slices packaged with the activated interleaves (Scannell et al. 2000). Also, when nisin immobilized in sodium caseinate films was tested against *Listeria* inoculated on the surface and in depth on mini red Babybel soft cheese, the presence of the active film resulted in a 1.1 log CFU/g reduction in *L. innocua* counts on the cheese surface after 1 week of storage at 4 °C as compared to control samples (Cao-Hoang et al. 2010). Inactivation rates decreased as depth of inoculation increased, e.g. 1.1, 0.9 and 0.25 log CFU/g for distances from the contact surface of 1, 2, and 3 mm, respectively, reflecting the nisin diffusion gradient. The study concluded that nisin immobilized in sodium caseinate films a promising method to overcome problems associated with post-process contamination, thereby extending the shelf life and possibly enhancing the microbial safety of cheeses.

Added lacticin 3147 powder rapidly inactivated *L. monocytogenes* and reduced *S. aureus* viable cell counts in an infant milk formulation, and was highly effective against *L. monocytogenes* in natural yogurt and in Cottage cheese (Morgan et al. 2001). Addition of 10 % lacticin 3147 powder reduced the concentration of viable *Listeria* below detectable levels in yogurt within 60 min or killed 85 % of cells in cottage cheese within 120 min (Morgan et al. 2001). However, optimisation of lacticin 3147 powder to increase specific activity may be necessary in order to decrease the amount of added powder required for an effective microbial inhibition.

Several enterococal bacteriocins have been tested for preservation of dairy foods (Giraffa 1995; Foulquié Moreno et al. 2006; Gálvez et al. 2008). Enterocins CCM 4231, CRL35, or AS-48 can reduce the levels of *L. monocytogenes, S. aureus* or *B. cereus* in dairy products. A concentrated enterocin CRL35 preparation added to goat cheese (10,400 AU/ml) reduced the population of *L. monocytogenes* by 9 log units by the end of ripening period without affecting the cheese quality (Farías et al. 1999). Lauková et al. (1999) reported that addition of enterocin CCM 4231 (3,200 AU/ml) in yogurt milk inoculated with *L. monocytogenes* reduced the levels of listeria after 24 h incubation at 30 °C by about 2 log CFU/ml. Similarly, enterocin addition (3,200 AU/ml) to skim milk decreased the viable counts of *S. aureus* from 10 log CFU/ml to 2 log CFU/ml after 24 h incubation of milk at 27 °C. This bacteriocin also reduced the levels of *Listeria* in "bryndza" (a traditional Slovak soft cheese from sheep milk) and Saint-Paulin cheese (Lauková et al. 2001; Lauková and Czikková 2001) but it did not achieve complete elimination of the bacteria. For example, addition of enterocin CCM 4231 during Saint-Paulin cheese preparation (3,200 AU/ml) reduced the population of inoculated *L. monocytogenes* by almost 5 log cycles for up to 1 week, followed by proliferation of the listeria afterwards (Lauková et al. 2001).

Bcteriocins from enterococci have been used to prepare activated films or coatings for use in the cheese industry. In one study, Iseppi et al. (2008) tested the anti-

listerial effects of film packagings activated with enterocin 416K1 on fresh cheese surfaces. In the fresh soft cheese samples packed in enterocin-activated film, the listeria counts were lower than controls by about 1 log unit up to 28 days for samples stored at 4 °C and for up to 7 days for samples stored at 22 °C. This approach for application of bacteriocins not only reduces the levels of listeria on cheese surfaces but is also an effective barrier against cross-contamination of the cheeses.

Enterocin AS-48 has been suggested for biopreservation of prepared dishes and desserts containing milk. In boiled rice and in a commercial rice-based infant formula dissolved in whole milk inoculated with vegetative cells or with endospores of *B. cereus,* enterocin AS-48 (20–35 μg/g) reduced viable cell counts below detectable levels during storage for up to 15 days in a temperature range of 6–37 °C and prevented enterotoxin production (Grande et al. 2006a). Bacteriocin activity was improved by adding sodium lactate, decreasing the effective bacteriocin concentration to 8–16 μg/g). Although the bacterial endospores were resistant to this bacteriocin, application of AS-48 in combination with heat treatments decreased the thermal death *D* values for endospores (Grande et al. 2006b). In desserts and bakery ingredients, the bactericidal effect of AS-48 on *S. aureus, B. cereus* and *L. monocytogenes* depended on the food substrate and the target bacteria (Martínez Viedma et al. 2009a, b). The lowest and highest efficacies were always detected in soy-based desserts and in gelatin pudding, respectively. *L. monocytogenes* was completely inactivated by bacteriocin concentrations in the range of 5–25 μg/g, depending on the substrate, and *B. cereus* was inactivated in a range of 15–50 μg/g. Bacteriocin addition to gelatin pudding prevented the production of proteases by *B. cereus* and the consequent gelatin liquefaction. Inactivation of *S. aureus* required a higher bacteriocin concentration (50 μg/g) and also a lower population density of staphylococci, not higher than 5 log CFU/g. The bacteriocin also showed a variable degree of activity against *S. aureus* in substrates like pumpkin confiture, diluted almond cream or liquid caramel, but was ineffective in vanilla or chocolate creams (Martínez Viedma et al. 2009). In chocolate cream, where higher bacteriocin concentrations were required because of interaction of the bacteriocin with the food substrate, antimicrobial activity increased markedly when AS-48 was tested in combination with eugenol, 2-nitropropanol or Nisaplin.

Pediocin PA-1/AcH preparations are interesting for application in dairy products due to the bacteriocin antilisterial activity, stability in aqueous solutions at ambient temperature and also during freezing and heating, and wide pH range for activity (Nes et al. 1996; Rodríguez et al. 2002). The commercial preparations containing pediocin in the form of Alta™ products can be used as ingredients in dairy foods. Several studies have shown that added pediocin PA-1/AcH is effective in reducing the levels of *L. monocytogenes* in several types of dairy products such as dressed Cottage cheese, half-and-half cream, and cheese sauce (reviewed by Rodríguez et al. 2002).

Other bacteriocins of interest in preservation of dairy foods are the propionicins. Propionibacteria are used in some dairy fermentations and may produce bacteriocins with broad inhibitory spectra (Holo et al. 2002). Microgard™ is a commercial preparation containing an antimicrobial peptide produced by *Propionibacterium*

freudenreichii ssp. *shermanii* (Weber and Broich 1986), which is approved in certain countries for commercial use as an ingredient mainly in dairy products such as Cottage cheese and yogurt. Bacteriocins produced by *Propionibacterium jensenii* P126 and P1264 strains have been patented as anti-bacterial agents for controlling the growth of certain lactic acid bacteria. These bacteriocins could be particularly useful in controlling the over-acidification of yogurt to decrease the sour taste often found in this product.

Some bacteriocins from bacteria not associated with milk fermentations been investigated for application in dairy foods. Variacin in the form of a dry milk-based ingredient inhibited the proliferation of *B. cereus* in chilled dairy products, vanilla and chocolate desserts (Mollet et al. 2004). There is also a growing interest in exploitation of bacteriocins from bacilli. Cerein 8A is an antimicrobial peptide produced by the soil isolate *B. cereus* 8A, with bactericidal activity towards *L. monocytogenes* and *B. cereus* (Bizani et al. 2005). Cerein addition inhibited growth of *L. monoytogenes* in milk and on the surface of Minas-type cheese during refrigeration storage, suggesting its potential use as biopreservative in dairy products.

5.2 Application of Bacteriocin-Producing Strains

5.2.1 Inhibition of Foodborne Pathogens

L. monocytogenes is considered the main foodborne pathogen of concern in cheese and dairy products. Therefore, many different studies have focused on the application of antilisterial starter or adjunct cultures for inhibition of this bacterium (Table 5.1). Nisin-producing lactococcal strains inhibit *L. monocytogenes* in several types of cheeses such as Cottage, Camembert or Manchego cheese made from raw milk. They can also reduce *S. aureus* viable counts (Deegan et al. 2006; Gálvez et al. 2008), but often lack the technological properties required for cheese making such as fast acidification capacity and proteolytic activity. For this reason, they should be recommended as adjunct cultures in combination with suitable nisin-resistant strains as the primary starters. Nevertheless, a comparative study on performance of bacteriocin-producing *Lactococcus lactis* strains selected on technological criteria for Cottage cheese fermentation (one nisin Z producer, one nisin A producer and two lacticin 481 producers) established that the nisin A producing *L. lactis* 40FEL3, and to a lesser extent the lacticin 481 producers 32FL1 and 32FL3, successfully controlled the growth of the pathogen during the manufacture and storage of Cottage cheese (Dal Bello et al. 2011).

The lactococci can also produce other bacteriocins (such as lacticin 3147or lacticin 481) in fermented dairy products (Guinane et al. 2005). Lacticin 3147-producing starter cultures have been tested to control the non-starter lactic acid bacteria population in Cheddar cheese (Ryan et al. 1996). In order to develop suitable starters, the plasmid coding for lacticin 3147 production was transferred to suitable recipient

lactococci. Food-grade lactococcal starters which produce the lantibiotics lacticin 3147 and lacticin 481 have also been reported (O'Sullivan et al. 2003). Lacticin production by lacticin 3147 modified starters successfully inhibited *L. monocytogenes* in Cottage cheese, in semi-hard raw-milk cheeses and on the surface of a mould-ripened cheese and smear-ripened cheese (O'Sullivan et al. 2006). In Cottage cheese inoculated with a *L.lactis* transconjugant strain, the bacteriocin concentration in the curd reached 2,560 AU/ml, and bacteriocin activity could be detected throughout the 1 week storage period. In cottage cheese samples held at 4 °C, there was at least a 99.9 % reduction in the numbers of *L. monocytogenes* Scott A in the bacteriocin-containing cheese within 5 days, whereas in the control cheeses, numbers remained essentially unchanged. At higher storage temperatures, the kill rate was more rapid (McAuliffe et al. 1999). In a smear-ripened cheese, applications of a live lacticin 3147-producing culture on a cheese surface containing *Listeria,* the viable cell concentrations of the pathogen were found to be up to 100-fold lower than in the cheese treated with a bac- *L. lactis* strain as control. The lactococci have also been tested for heterologous production of other bacteriocins such as enterocin A. The resulting starter derivative successfully controlled the levels of *L. monocytogenes* during Cottage cheese fermentation (Liu et al. 2008).

Enterococci are well adapted to grow in milk, and can produce sufficient bacteriocin amounts in milk substrates as to inhibit pathogenic bacteria such as *L.monocytogenes* and others. For example, *E. faecalis* EJ97 produced enterocin EJ97 during cocultivation in half-skimmed milk, although its capacity to control *L. monocytogenes* was limited to listerial populations of low densities ($\leq 10^3$ CFU/ml; García et al. 2004). *Enterococcus faecium* strain F58, isolated from Moroccan jben goat's cheese, was also able to produce bacteriocin in milk and to achieve a partial inhibition of *L. monocytogenes* during cocultivation in goat milk (Achemchem et al. 2006). In addition, pre-cultivation of strain F58 in milk for 12 h before inoculation of the listeria (at 3 log CFU/ml) produced enough bacteriocin to completely inactivated the inoculated listeria during further incubation. The enterocin AS-48 producer strain *E. faecalis* A-48-32 was able to produce enough bacteriocin in nonfat cow milk to reduce the population of co-inoculated *B. cereus* below detectable levels after 72 h of cocultivation at 30 °C (Muñoz et al. 2004). In cocultures done with skim milk, this strain was also able to control *S. aureus* (Muñoz et al. 2007).

Enterococci are very often found as part of the adventitious microbiota in fermented foods, including many traditional cheeses, and exhibit many biochemical properties of technological interest in dairy fermentations such as production of organic acids and acidification, proteolytic and peptidolytic activities, lipolytic and esterase activities, and citrate and pyruvate metabolism, together with their capacity to produce bacteriocins (Giraffa 2003). Bacteriocin-producing enterococci have been investigated as adjunct cultures for cheese making because of their robustness, natural presence in cheeses, and production of several bacteriocins with strong antilisterial activity (Giraffa 1995; Foulquié Moreno et al. 2003; Franz et al. 2007; Gálvez et al. 2008). Inoculation of Jben goats' milk cheese with bacteriocinogenic strain *E. faecium* F58 as an adjunct culture, caused a sharp decrease in the concentration of viable *L. monocytogenes*, which were undetectable after 1 week of cheese

storage at 22 °C (Achemchem et al. 2006). The bacteriocinogenic strain of *E. faecium* 7C5 (which produces enterocin AS-48), added as an adjunct with a thermophilic culture in soft cheese, led to complete death of *L. monocytogenes* and *L. innocua* without altering the acidifying activity of the starter culture (Giraffa et al. 1995b). When tested in cheeses, other strains producing enterocin AS-48 showed strong inhibition of *L. monocytogenes*, as well as *B. cereus* and *S. aureus* (Núñez et al. 1997; Muñoz et al. 2004, 2007). During the manufacture of Manchego cheese made from raw ewe's milk, *E. faecalis* INIA 4 was able to compete with cheese microbiota and produce enterocin (Núñez et al. 1997). In cheeses inoculated with INIA 4 strain, *L. monocytogenes* Ohio counts were reduced below 1 log CFU/g in the ripened cheese for up to 60 days. However, *L. monocytogenes* Scott A did not seem to be affected by the inoculated enterocin-producer in the cheese. In a separate study, when the enterocin AS-48 producer strain *E. faecalis* A-48-32 was co-inoculated with *B. cereus* during cheese manufacture, viable cell counts of the bacilli were 5.6 log units lower than controls after 30 days of ripening (Muñoz et al. 2004). The efficacy of strain A-48-32 against *S. aureus* was lower compared to *B. cereus*, but staphylococci counts in treated cheeses remained at least 1 log CFU/g below controls throughout at least 1 month storage (Muñoz et al. 2007).

Enterococcus faecium RZS C5 (a natural cheese isolate carrying the structural genes for enterocins A, B and P) was reported to be effective as an anti-listeria bacteriocin-producing co-culture in Cheddar cheese manufacture. The strains *Enterococcus mundtii* CRL35 and *E. faecium* ST88Ch isolated from cheeses were tested for their capability to control growth of *L. monocytogenes* 426 in experimentally contaminated fresh Minas cheese during refrigerated storage (Vera Pingitore et al. 2012). Growth of *L. monocytogenes* 426 was inhibited in cheeses containing *E. mundtii* CRL35 up to 12 days at 8 °C, stressing the potential of this strain for application in Minas cheese. However, *E. faecium* ST88Ch was less effective in the control of listeriae.

Strains of enterococci and lactococci producing bacteriocins (such as enterocins I, TAB 7, TAB 57, AS-48, nisin A, nisin Z and lacticin 481) have been tested in combination with HHP treatments with the aim to improve the safety of cheeses made from raw milk. Inoculation of milk with bacteriocinogenic strains before cheese making followed by application of HHP treatment to the cheeses was reported to increase the bactericidal activity against *L. monocytogenes, S. aureus* and *E. coli* O157:H7. For example, for 300 MPa treatment (10 min), counts of *L. monocytogenes* were always lower in the cheeses inoculated with the bacteriocin-producing enterococci both on day one after treatment and also after 60 days of ripening (at 12 °C under vacuum). For *S. aureus*, inoculation of cheeses with bacteriocin-producing enterococci also improved the lethal effects of HHP treatments (Arqués et al. 2005b). When similar treatments (300 MPa, 10 min; 500 MPa, 5 min) were applied to cheeses challenged with *E. coli* O157:H7, the reductions obtained for the treatment at 300 MPa were in the range of 0.7–2 log CFU/g on day one after treatment and 0.2–1.2 log CFU/g after 60 days as compared to the controls not inoculated with bacteriocin producers (Rodríguez et al. 2005). For 500 MPa, greatest differences were observed at day one after treatment, with in which no viable

cells were detected in any of the cheese samples inoculated with bacteriocin producers. The authors concluded that the application of reduced pressures combined with bacteriocin-producing enterococci can improve cheese safety while decreasing the deleterious effects on cheese quality caused by HHP at higher pressures.

During the manufacture of smear-ripened cheeses, smear operations increase the risk for surface contamination and cross-contamination with *L. monocytogenes*. In the process of Taleggio smear cheese making, inoculated *E. faecium* 7C5 was reported to produce bacteriocin (Giraffa et al. 1995a) and to inhibit the growth of *L. monocytogenes* Ohio on the cheese surface (Giraffa and Carminati 1997). When *E. faecium* WHE 81, a multi-bacteriocin producer isolated from Munster cheese, was inoculated on the cheese surface during smearing operation, further inoculation of the cheeses with a low level inoculum of *L. monocytogenes* (50 CFU/g) resulted in suppression of the listeria or its complete growth inhibition compared with the controls inoculated with a bac$^-$ strain (Izquierdo et al. 2009). The inoculated enterococci had no detrimental effects on the on pH, fungal flora or pigmented bacteria in the cheese rind during ripening.

Thermophilic streptococci are important as dairy starters used in large scale in the production of yogurt and certain cheese varieties. The bacteriocin-producer strain *Streptococcus salivarius* subsp. *thermophilus* B was tested as a thermophilic starter in yogurt to control *L. monocytogenes* and *S. aureus.* The counts of *Listeria* were reduced below detectable levels, but the staphylococci survived in the produced yogurt. Use of the bac + starter was reported to extend the product shelf-life by 5 days (Benkerroum et al. 2002).

Pediococci are not well adapted to dairy substrates, due to their lack or very slow lactose fermentation activity (Papagianni and Anastasiadou 2009). However, some strains such as *Pediococcus acidilactici* NRRL-B-18925 are particularly effective in producing bacteriocin in milk based media. Since pediocin PA-1/AcH is not inhibitory to bacterial species employed as yogurt starters (Gonzalez and Kunka 1987) there is a great interest for application of producer strains in developing more naturally preserved yogurts and to avoid proliferation of cross-contaminating pathogens during yogurt processing. Vedamuthu Ebenezer (1995) patented a method for producing a yogurt product which contains bacteriocin active against undesirable microbiota. The yogurt product can be dried for use in various foods. Pediocin production in milk has been reported in coculture with yogurt starter cultures, at the expense of the excess sugar released from lactose hydrolysis by the starters (Somkuti and Steinberg 2010). While no bacteriocin production was detected when *P. acidilactici* was inoculated into milk as a monoculture, when grown in combination with the yogurt starter cultures *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, pediocin concentration reached 3,200–6,400 units/ml after 8 h of incubation.

The development of pediocin-producing genetically-engineered *L. lactis* strains can be another approach to solve the problems associated with the use of pediococci in dairy substrates. Pediocin-producing lactococci have shown significant potential for inhibition of foodborne pathogens in cheeses. Examples are recombinant *L. lactis* MM217 as a pediocin-producing starter culture in Cheddar cheese (Buyong et al.

1998) or strains of *L. lactis* ESI 153 and *L. lactis* ESI 515, isolated from hand-made raw milk cheese and transformed into pediocin producers (Reviriego et al. 2005). The pediocin-producing transformants reduced viable counts of *L. monocytogenes* in cheese below 50 or 25 CFU/g at the end of the ripening period.

Bacteriocin-producing lactobacilli have also been suggested for preservation of dairy foods. *Lactobacillus plantarum* WHE 92 is a spontaneous pediocin producer that grows well and produces satisfactory pediocin concentrations in Munster cheese (Ennahar et al. 1996). *L. plantarum* LMG P-26358 isolated from a soft French artisanal cheese produces plantaricin 423, which has strong anti-*Listeria* activity. This relatively narrow spectrum bacteriocin also exhibited antimicrobial activity against species of enterococci, but did not inhibit dairy starters including lactococci and lactobacilli. A strong listericidal effect was detected in cheeses made with *L. plantarum* LMG P-26358 as an adjunct culture in combination with a nisin producer, and the single inoculation with LMG P-26358 performed even better than the single nisin producer. Combination of strain LMG P-26358 as adjunct culture with nisin-producing cultures may be an effective strategy to improve the safety and quality of dairy products (Mills et al. 2011). Another interesting example is the human isolate probiotic strain *Lactobacillus gasseri* K7, producer of bacteriocins with wide range of inhibition (Čanžek Majhenič et al. 2003). Application of bacteriocin-producing probiotic strains could be exploited with the purpose of improving food safety and quality and at the same time providing health benefits.

5.2.2 *Inhibition of Bacteria Producing Gas-Blowing Defects in Cheeses*

Gas production is an undesirable defect in most cheeses, and may be caused by outgrowth of *C. tyrobutyricum* (and other clostridia such as *Clostridium sporogenes, Clostridium beijerinckii* or *Clostridium butyricum*) spores surviving heat treatments applied to milk before processing (Cocolin et al. 2004; Le Bourhis et al. 2007), and also by some heterofermentative LAB. Species of *Clostridium* can ferment lactic acid with production of butyric acid, acetic acid, carbon dioxide and hydrogen. Butyric acid fermentation (also known as late blowing defect) is one of the major causes of spoilage in semi-hard and hard ripened cheeses. This fermentation originates texture and flavor defects in the cheeses, causing important economic losses in the cheese industry (McSweeney and Fox 2004). Bactofugation, milk ultrafiltration, and addition of nitrite or lysozyme are often applied to prevent butyric acid fermentation in cheeses, but also the inoculation of milk with bacteriocinogenic lactic acid bacteria (LAB) in cheese manufacture can provide satisfactory results (Table 5.1). Application of nisin-producing starter cultures to prevent gas-blowing defects in cheese was proposed as early as 1951. Nisin-producing strains have been used for developing a starter culture system for manufacture of Cheddar cheese (Roberts et al. 1992) and Gouda cheese (Bouksaim et al. 2000), among others. Strains producing the natural variant nisin Z have also shown to reduce the

levels of *C. tyrobutyricum* in cheese. For example, *L. lactis* ssp. *lactis* IPLA 729, a nisin Z producer isolated from raw milk cheese, was able to produce nisin Z in semi-hard Vidiago cheese. Nisin Z activity reached a concentration of 1,600 AU/ml in 1-day cheeses and this level was maintained until 15 days of ripening. The produced nisin reduced the levels of a *C. tyrobutyricum* spoilage strain inoculated in the cheeses by ca. 3 log cycles, while considerable bacterial growth was observed in control cheeses inoculated with a commercial starter culture and supplemented with nitrate (Rilla et al. 2003). In another study, when Manchego cheese artificially contaminated with endospores of *C. beijerinckii* was inoculated with a *L. lactis* strain producing nisin and lacticin 481 as a starter, no late-blowing defects were observed after 120 days of ripening, and the concentrations of lactic acid and volatile compounds were similar to control cheese (Garde et al. 2011).

Lacticin 3147-producing lactococci have been shown to inhibit *C. tyrobutyricum* spores and prevent late blowing in semi-hard cheeses, and also demonstrated a considerable inhibition of heterofermentative lactobacilli and their associated blowing defects in cheese (Martínez-Cuesta et al. 2010). The authors suggested that application of lacticin producers in cheese manufacture is a promising alternative to the addition of lysozyme, given the increasing concerns about the potential allergenicity of this additive in egg allergic consumers.

Thermophilic streptococci also have a potential for inhibition of *C. tyrobutyricum* in some cheeses. The bacteriocin thermophilin from *S. thermophilus* ST580 is active against *C. tyrobutyricum,* but not against the thermophilic lactobacilli used as starters. Curds made with strain ST580 and inoculated with *C. tyrobutyricum* endospores showed no gas production for up to 20 days (Mathot et al. 2003). This strain could be included in thermophilic starters for hard cheese making. *Streptococcus macedonicus* ACA-DC 198, which produces the food-grade lantibiotic macedocin, could also be employed to inhibit gas formation in cheese (De Vuyst and Tsakalidou 2008). When tested as an adjunct culture in Kasseri cheese production, this strain produced macedocin in the cheese and inhibited outgrowth of *C. tyrobutyricum* spores during the cheese production and ripening (Anastasiou et al. 2009).

Among lactobacilli, the bacteriocin producer strain *L. gasseri* K7 was able to survive during semi-hard-type cheese manufacturing, and reduced outgrowth of *C. tyrobutyricum* and butyric acid formation in the cheeses (Bogovič Matijašić et al. 2007).

5.2.3 Improving Cheese Quality

Most bacteriocins act on the bacterial cytoplasmic membrane, modifying cell permeability. A secondary effect of bacteriocins is the induction of cell lysis, as a result of deregulation of cell wall autolysins (Gálvez et al. 1990). This effect was further observed on dairy starter cultures, and led to more detailed studies on the potential applications for the release of bacterial intracellular enzymes (such as lipases, proteases, peptidases, and amino acid-converting enzymes) of technological relevance

in cheese ripening (Lortal and Chapot-Chartier 2005; Peláez and Requena 2005; Deegan et al. 2006).

In one study, *L. lactis* DPC3286 (producing lactococcins A, B, and M) was tested as an adjunct starter in cheddar cheese manufacture to induce lysis of a sensitive acidifying starter strain (Morgan et al. 1997) or in combination with a bacteriocin-resistant starter. In experimental cheeses with the bacteriocin-resistant starter, the levels of free amino acids increased together with a greater release of the intracellular enzyme lactate dehydrogenase (LDH), and the cheeses showed lower bitterness compared to controls (Morgan et al. 2002). Because lactococcins A, B, and M have a narrow spectrum of activity limited to lactococci, their producer strains could be applied specifically for acceleration of cheese ripening (Ross et al. 1999). Another study reported that addition of the nisin Z-producing strain *L. lactis* ssp. *lactis* biovar *diacetylactis* UL719 during cheddar cheese making increased lipolysis and proteolysis, as well as the formation of hydrophilic and hydrophobic peptides, and enhanced the sensory characteristics of cheese (Benech et al. 2003). Strain UL719 was also tested for acceleration of autolysis of an adjunct *L. delbrueckii* subsp. *bulgaricus* strain, with the result of increasing cheese proteolysis and improving the cheese texture (Sallami et al. 2004). An interesting observation was that bacteriocin production by strain UL719 in cocultures with nisin-sensitive starters *Lactobacillus rhamnosus* RW-9595M and *L. lactis* subsp. *cremoris* was stimulated by 3.1- to 4.6-fold. This stimulation was attributed to the high proteolytic activity of *L. cremoris* and to the release of intracellular nutrients due to autolysis and nisin Z-induced lysis (Grattepanche et al. 2007).

Production of lacticin 481 by *L. lactis* subsp. *lactis* strain DPC5552 induced release of the intracellular enzymes LDH and postproline dipeptidyl aminopeptidase by starter strain *L. lactis* HP without completely inhibiting its growth (O'Sullivan et al. 2002a). Bacteriocin production also induced the release of elevated levels of LDH from the starter without severely compromising its acid-producing capabilities in a cheddar cheese–making trial. Further studies indicated that lacticin 481 was also able to accelerate starter cell lysis (O'Sullivan et al. 2003), and that lacticin 481-producing cultures promoted early lysis of *Lactobacillus helveticus* cells in Hispánico cheese and increased the proteolytic activity (Garde et al. 2006). During the Hispánico cheese–making process, inoculation of milk with strain *L. lactis* subsp. *lactis* INIA 415 (harboring the structural genes of lacticin 481 and nisin Z production) promoted early lysis of mesophilic and thermophilic starter bacteria and increased extracellular aminopeptidase activity (Garde et al. 2002). It also lowered the ratio of hydrophobic-to-hydrophilic peptides, increased the free amino acid content (Avila et al. 2006), and enhanced the formation of several volatile compounds of relevance to the odor and aroma of the cheese, such as hexanal, 2-methyl-1-propanol, 3-methyl-1-butanol, acetone, 2-pentanone, 2-hexanone, and 2-heptanone, but decreased the formation of acetaldehyde, ethanol, 3-methyl-3-buten-1-ol, 3-methyl-2-buten-1-ol, ethyl acetate, ethyl butanoate, ethyl hexanoate, 2-butanone, 2,3-butanedione, 2,3-pentanedione, and 3-hydroxy-2-butanone (Garde et al. 2005).

Lacticin 3147 production in cheese also induced bacterial lysis of cheese starters, increased cheese proteolysis and facilitated the access of bacterial amino-acid converting enzymes to amino acids (Deegan et al. 2006). The accelerated starter cell lysis enhanced reactions such as isoleucine transamination, increasing the formation of alpha-keto-beta-methyl-n-valeric acid and 2-hydroxy-3-methyl-valeric acid and cheese aroma intensity due to the higher 2-methylbutanal formation. Optimisation of aroma production could be achieved by selective combination of starters, such as a lacticin 3147-producing *L. lactis* transformant in combination with adjunct cultures producing aminotransferase and a-keto acid decarboxylase activities (Fernández de Palencia et al. 2004).

The application of bacteriocin-producing adjunct cultures to accelerate cheese ripening can be a cheaper approach compared to the addition of exogenous lytic enzymes (Papagianni and Anastasiadou 2009). Application of bacteriocins and/or their producer strains in the development of stabilised cheese flavouring systems has been patented (Dias et al. 2009). Using microbial cells with high aminopeptidase activity in combination with an antimicrobial that can permeabilize the cells can decrease the levels of aminopeptidases that need to be added to the matrix, thereby increasing efficiency. Cheeses produced at the expense of enzymes released form the bacterial cells have a more rounded flavour (Dias et al. 2009). An adjunct *P. acidilactici* culture that accelerates and enhances flavour formation in Cheddar and semi-hard cheeses due to the production of bacteriocins has been marketed by Danisco (CHOOZIT™ Lyo. Flav 43).

Another suggested application of bacteriocin-producing cultures is the inactivation of adventitious non-starter LAB (NSLAB) microbiota during cheese ripening. NSLAB can proliferate during ripening and often tend to become the dominant microbiota in the cheese. The precise role of NSLAB strains in flavor development remains unclear, but they certainly contribute in the quality of many cheeses (Fox et al. 1998). Control of NSLAB is still a pending issue in dairy industries. Growth of NSLAB may induce batch to batch variations in the sensory quality of cheese and cause defects such as the formation of calcium lactate crystals (due to racemation of L-lactate to D-lactate), slit formation and off-flavour development, but they may also exert beneficial effects on the cheeses. Application of lacticin 3147-producing starters has been proposed as a way to enhance cheese quality through inhibition of adventitious NSLAB microbiota during ripening (Ryan et al. 2001; Deegan et al. 2006). Co-inoculation of a lacticin-3147 adapted *Lactobacillus paracasei* subsp. *paracasei* adventitious strain (isolated from a well-flavored, commercial Cheddar cheese) with a lacticin-3147 producing starter culture allowed a better control of NSLAB microbiota during ripening. By using randomly amplified polymorphic DNA-PCR, it was demonstrated that the resistant adjunct strain comprised the dominant microflora in the test cheeses during ripening (Ryan et al. 2001). During Cheddar cheese manufacture, ripening can be accelerated by increasing the temperature from 7 to 12 °C, but this also results in a higher risk of spoilage due to a more rapid proliferation of NSLAB. Inoculation with a lacticin 3147-producing strain allowed a better control of NSLAB during cheese ripening at elevated temperature. Lacticin 481 production has also shown to reduce the concentrations of

NSLAB in cheese by 4 to 2 orders of magnitude during ripening. Bacteriocin production in the cheese resulted in selection of NSLAB that were much more resistant to the bacteriocin than isolates from control cheeses. Therefore, it would be possible to select bacteriocin-resistant strains that do not have negative effects on cheese ripening as the predominant NSLAB.

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Chapter 6
Biopreservation of Egg Products

6.1 Application of Bacteriocins

A few studies have investigated the preservation of egg and egg products by application of bacteriocins (Table 6.1). The commercial use of liquid whole egg requires processing in order to prolong its shelf-life and to inactivate foodborne pathogens. As an alternative to conventional pasteurization, an ultrapasteurization processes (i.e., heating at >60 °C for <3.5 min) was developed, which, when coupled with aseptic processing and packaging, produced liquid whole egg with a shelf life of at least 10 weeks at 4 °C. The use of effective aseptic filling and packaging systems (to prevent postpasteurization contamination) remains an essential component in the production of ultrapasteurized liquid whole egg with an extended pathogen-free shelf life. Contrary to *Salmonella*, conventional minimal egg pasteurization processes do not grant a complete inactivation of *Listeria monocytogenes*. As a matter of fact, *Listeria* species can be isolated from commercially broken raw liquid whole egg. Therefore, it was proposed to use bacteriocins for the control of *Listeria* in this food system (Schuman and Sheldon 2003). Addition of nisin to pasteurized liquid whole egg reduced the viable counts of *L. monocytogenes*, increased the product refrigerated shelf-life, and protected the liquid egg from growth of *L. monocytogenes* and *Bacillus cereus* during storage (Delves-Broughton et al. 1992; Knight et al. 1999; Schuman and Sheldon 2003). Nisin (200 IU/ml) extended the shelf life of conventionally pasteurized liquid whole egg at 6 °C by 9 to 11 days relative to nisin-free control samples (Delves-Broughton et al. 1992). The addition of nisin (1,000 IU/ml) to pH-adjusted ultrapasteurized liquid whole egg reduced *L. monocytogenes* populations by 1.6 to over 3.3 log CFU/ml and delayed (pH 7.5) or prevented (pH 6.6) the growth of the pathogen for 8–12 weeks at 4 and 10 °C (Schuman and Sheldon 2003). Both nisin and pediocin PA-1/Ach acted synergistically with heat treatments against *L. monocytogenes* (Knight et al. 1999; Muriana 1996). Nisin added at 10 mg/l significantly decreased the decimal reduction times (D-values) for *L. monocytogenes* in liquid whole egg. This effect was greater when the bacteriocin

A. Gálvez et al., *Food Biopreservation*, SpringerBriefs in Food, Health, and Nutrition, DOI 10.1007/978-1-4939-2029-7_6

Table 6.1 Examples of bacteriocin applications in egg products

Bacteriocin treatment	Effect(s)	Reference(s)
Nisin	Decreased the decimal reduction times (*D*-values) for *L. monocytogenes* in liquid whole egg	Knight et al. 1999
		Schuman and Sheldon 2003
	Inactivation of *L. monocytogenes* in pasteurized liquid whole egg	
	Improves product shelf life, inhibiting post-process proliferation of *L. monocytogenes* and *B. cereus*	
Nisin and PEF	Greater inactivation of *L. innocua* in liquid egg	Calderón-Miranda et al. 1999
Nisin and HHP	Greater inactivation of *L. innocua* and *E. coli* in liquid egg	Ponce et al. 1998
Nisin in polylactic acid (PLA) coating	Inactivation of *L. monocytogenes* in liquid egg white	Jin 2010
Nisin plus allyl isothiocyanate in PLA coating	Inactivation of a three-strain *S. enterica* cocktail in liquid egg white	Jin et al. 2013

was added at least 2 h before application of heat treatments (Knight et al. 1999). In spite of the fact that nisin is not active on Gram-negative bacteria, nisin addition also increased the heat sensitivity of *Salmonella Enteritidis* PT4 in liquid whole egg and in egg white during pasteurization (Boziaris et al. 1998).

The presence of *B. cereus* in liquid egg can pose a serious hazard to the food industry, since a mild heat treatment cannot guarantee its complete inactivation. In one study, the effects of added nisin, lysozyme, or a combination of both antimicrobials on the lag phase of *B. cereus* inoculated in homogenized liquid egg samples previously heated at 60 °C for 10 min to inactivate background microflora was investigated. The combination of lysozyme and nisin delayed the average onset of growth until 10 h at 25 °C or approximately 30 h in samples stored at 16 °C (Antolinos et al. 2011).

Bacteriocins have been tested in liquid egg in combination with pulsed electric field (PEF) and high hydrostatic pressure (HHP) treatments as a way to enhance inactivation of microorganisms. Exposure of *Listeria innocua* to nisin after PEF treatment at low temperature showed an additive to synergistic effect, depending on the bacteriocin concentration and the electric field intensity and number of pulses (Calderón-Miranda et al. 1999). PEF treatments sensitized *L.innocua* to further exposure to nisin in liquid whole egg. The PEF treatment followed by the exposure of *L. innocua* to nisin increased microbial inactivation compared to the inactivation observed when the bacterium was subjected to PEF alone. The combined effect of both factors (PEF treatment and nisin addition) was either additive or synergistic, depending on the intensity of PEF treatments (Calderón-Miranda et al. 1999). Addition of nisin in combination with HHP treatment markedly reduced viable cell counts of *Escherichia coli* and *L. innocua* in liquid whole egg (Ponce et al. 1998). A reduction of almost 5 log units in *E. coli* counts and more than 6 log units for *L.*

innocua was obtained at 450 MPa and 5 mg/l nisin. For this treatment, the two microorganisms were not detectable after 1 month of storage at 4 °C. However, nisin showed no effect in preventing growth of *E. coli* in samples stored at 20 °C after pressurization. Nevertheless, counts of *L. innocua* were about 5 log cycles lower than controls after 5 days of storage at 20 °C. This could be explained by the greater reduction of viable counts obtained for this bacterium in the combined treatment, but also to a post-process protective effect of the added nisin.

Another approach for application of nisin in egg was immobilization of the bacteriocin. Liquid egg white inoculated with *L. monocytogenes* Scott A was stored in glass jars that were coated with a mixture of polylactic acid (PLA) polymer and nisin, and stored at 4 and 10 °C (Jin 2010). *Listeria* cells in control and PLA coating without nisin samples declined 1 log CFU/ml during the first 6 days at 10 °C and during 28 days at 4 °C, and then increased to 8 or 5.5 log CFU/ml. In comparison, the treatment of PLA coating with 250 mg nisin rapidly reduced the cell numbers of *Listeria* in liquid egg white to undetectable levels after 1 day, and the bacterium remained undetectable throughout the whole storage periods (48 days at 10 °C and 70 days at 4 °C). Another study reported that a PLA coating containing allyl isothiocyanate in combination with 250 mg nisin reduced the population of a three-strain *Salmonella enterica* cocktail inoculated in liquid egg white to an undetectable level after 21 days of storage (Jin et al. 2013).

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Chapter 7
Biopreservation of Seafoods

7.1 Application of Bacteriocin Preparations

Listeria monocytogenes is the main bacterial pathogen of concern in seafood products. One study found *L. monocytogenes* in ca. 30 % of smoked-fish samples, although viable counts were below 100 CFU/g (Uyttendaele et al. 2009). Another study found populations of *L. monocytogenes* greater than 10^2 CFU/g in 2.6 % of fresh fish, 5.1 % in smoked fish and 10 % in salted-fish purchased in fish farms, while 20 % of smoked fish purchased in a fish market were also contaminated (Basti et al. 2006). The bacterium was also found in raw fillets of catfish (23.5 %), trout (5.7 %), tilapia (10.3 %), and salmon (10.6 %) (Pao et al. 2008), or in 44.5 % of raw freshwater fish tested (Yücel and Balci 2010). Bacteriocin preparations have been tested singly or in combination with other hurdles to control *L. monocytogenes* in different types of seafoods (Table 7.1).

7.1.1 Raw Seafoods

Inhibition of aerobic bacteria is important to prevent seafood spoilage. The combination of nisin and Microgard™ reduced the total bacterial counts and delayed growth of *L. monocytogenes* in fresh-chilled salmon during 14 days at 6 °C, increasing the product shelf life (Zuckerman and Ben Avraham 2002; Calo-Mata et al. 2008). The observed effect was explained by the inhibitory activity of Microgard™ on Gram-negative bacteria and nisin activity on Gram-positives. Inhibition of *L. monocytogenes* in fesh salmon was also considered to be relevant as a way of preventing or reducing the levels of this bacterium in processed products such as cold-smoked salmon. In another study, nisin (200 IU/g) added on fresh gilthead seabream fillets packed under modified atmosphere was the most effective treatment resulting in significant shelf life extension of fillets (48 days compared to 10 days

A. Gálvez et al., *Food Biopreservation*, SpringerBriefs in Food, Health, and Nutrition, DOI 10.1007/978-1-4939-2029-7_7

Table 7.1 Example applications of bacteriocin and bacteriocin-producing LAB in seafoods

Bacteriocin treatment	Effect(s)	Reference(s)
Nisin- radio frequency heating at 65 °C	Complete inactivation of *L. innocua* in sturgeon caviar or ikura	Al-Holy et al. 2004
Nisaplin	Inactivation of *L. monocytogenes* in red-pepper seasoned cod roe	Hara et al. 2009
Nisin-coated plastic films	Inactivation of *L. monocytogenes* in CSS during refrigeration storage	Neetoo et al. 2008a
	Inhibition of background spoilage microbiota	
Broad-spectrum bacteriocin preparation from *L. lactis* PSY2	Reduction of total viable counts on the surface of reef cod fillets	Sarika et al. 2012
Enterocin AS-48	Inhibition of biogenic amine forming LAB on sardine fillets	Ananou et al. 2014
C. divergens V41 or its culture supernatant	Inhibitory effect on *L. innocua* 2030c growth cold-smoked salmon-trout	Vaz-Velho et al. 2005
C. divergens M35, or divergicin M35	Suggested as bio-ingredient for application to the inactivation of *L. monocytogenes* in ready-to-eat seafood	Tahiri et al. 2009b
E. mundtii	Inhibition of of *L. monocytogenes* in CSS	Bigwood et al. 2012
L. curvatus CWBI-B28 culture, spraying with bacteriocin, packaging in bacteriocin-coated plastic film, cell-adsorbed bacteriocin	Variable inactivation of *L. monocytogenes* in CSS. Best results were reported for bacteriocin adsorbed on heat-inactivated cells	Ghalfi et al. 2006
L. curvatus ET30	Reduction of *L. innocua* counts on salmon fillets before and after cold-smoking and during vacuum pack storage	Tomé et al. 2008
Leuconostoc spp., *L. fuchuensis, C. alterfunditum*	Broad-spectrum bio-protective cultures	Matamoros et al. 2009
Bifidobacterium-thymol combination	Extended shelf life of fresh plaice fillets; inhibition of fresh packaged fish spoilers	Altieri et al. 2005
S. xylosus	Use as protective culture to decrease biogenic amine formation in salted and fermented anchovy	Mah and Hwang 2009
L. lactis (nisin-producer)	Use as starter culture to improve Senegalese guedj fish fermentation	Diop et al. 2009

for the control at 0 °C; Tsironi and Taoukis 2010). Also, when nisin was tested in combination with other antimicrobials (such as the lactoperoxidase system or with headspace CO_2 levels and EDTA), increased inactivation or growth delay of spoilage microbiota was observed in sardines and in fish muscle extract.

In one recent study, enterocin AS-48 was applied on sardine fillets immersion in a bacteriocin solution (250 mg/l) for 1 min (Ananou et al. 2014). The treated samples

were refrigeration stored under normal, vacuum, or modified atmosphere packaging. The application of enterocin AS-48 did not reduce the mesophilic, psychrotrophic, or Gram-negative bacteria viable cell counts under any of the storage conditions tested. AS-48 did cause significant reductions in viable staphylococci counts, especially under vacuum packaging. Storage of samples treated with enterocin AS-48 under modified atmosphere or under vacuum packaging allowed reductions (significant at some storage times) in histamine- and tyramine-forming LAB. The most interesting results of this study are those concerning the observed decrease (by several fold) in the levels of the biogenic amines cadaverine, putrescine, tyramine, and histamine determined after treatment with AS-48.

Antibacterial activity with broad inhibitory spectrum (*Arthrobacter* sp., *Acinetobacter* sp., *Bacillus subtilis*, *Escherichia coli*, *L. monocytogenes*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*) was detected in *Lactococcus lactis* PSY2 isolated from the body surface of marine perch (Sarika et al. 2012). Surface-application of a bacteriocin preparation derived from this strain on fillets of reef cod reduced bacterial growth during storage at 4 °C. The total viable count revealed that PSY2-treated fish samples remained within the maximum limit of acceptability (10^7 counts/g, according to International Commission of Microbiological Standards for Foods 1986) until day 21, while the untreated controls became unacceptable before the 14th day of storage (Sarika et al. 2012). The maximum inhibitory effect of bacteriocin PSY2 was observed against *Staphylococcus* sp. and *Pseudomonadaceae* which were reduced by 1.8 and 3.37 log units in the PSY2 treated fillets compared to the control. Acceptability in terms of sensory attributes was significantly higher in the bacteriocin-treated samples.

Data on bacterial food poisoning associated to consumption of seafoods are comparatively more scarce compared to data on incidence of *L.monocytogenes*. However, a recent study reported that enterotoxigenic *Bacillus cereus* can grow on the surface of fresh salmon at abusive temperatures, with generation times of 169.7, 53.5, and 45.6 min were at 12, 16, and 20 °C (Labbé and Rahmati 2012). Nonhemolytic enterotoxin was detected on salmon after 20 h at 20 °C and after 26 h at 16 °C when levels of *B. cereus* were in excess of 10^8 CFU/g, indicating that fresh salmon can serve as an excellent substrate for enterotoxigenic *B. cereus* and that this organism can reach levels associated with foodborne illness following moderate temperature abuse (Labbé and Rahmati 2012). Nisin, at concentrations of 1 and 15 mg/g of salmon, reduced the levels of *B. cereus* by 2.5- and 25-fold, respectively after 48 h incubation at 16 °C, although the effect of added nisin on enterotoxin production was not reported (Labbé and Rahmati 2012).

7.1.2 Ready-to-Eat Seafoods

Minimally processed refrigerated ready-to-eat seafoods can pose health risk to susceptible individuals due to contamination by *L. monocytogenes*. Proliferation of *L. monocytogenes* in slightly processed products which are consumed without

further cooking (such as cold-smoked seafood products) is a matter of concern, and therefore extensive work has been carried out on application of bacteriocins in this field (O'Sullivan et al. 2002; Chen and Hoover 2003; Drider et al. 2006; Calo-Mata et al. 2008; Galvez et al. 2008). Several bacteriocins (such as nisin, psicocins, divercin or sakacin P) have been tested, either by addition of bacteriocin preparations (either by immersion, spraying, or mixing with food matrix) bacteriocin injection, or immobilisation on plastic films or coatings. In vacuum-packaged CSS stored at 10 °C for 3 weeks, purified sakacin P added at 12 ng/g or 3.5 µg/g partially or completely inhibited growth of *L. monocytogenes* (Aasen et al. 2003). It was also observed that bacteriocin titres in salmon tissue decreased during storage, which was attributed to proteolytic degradation of the bacteriocin.

Duffes et al. (1999a) tested the effect of adding semipurified divercin V41 from *Carnobacterium divergens* V41 (isolated from trout intestine) and piscicolins from *Carnobacterium piscicola* V1 on *L. monocytogenes* inoculated in CSS stored at 4 or 8 °C. Crude extracts of piscicocins were bactericidal at 4 °C and 8 °C, while divercin V41 inhibited (4 °C) or delayed (8 °C) growth. In CSS stored at 10 °C, purified divergicin M35 (50 µg/g) as well as concentrated culture supernatants reduced viable counts of *L. monocytogenes* by 1 log CFU/g at the beginning of storage and inhibited or retarded growth for up o 21 days (Tahiri et al. 2009a, b). Total lactic acid bacteria counts were not affected by bacteriocin addition. Vaz-Velho et al. (2005) applied a different strategy, based on immersion of salmon-trout fillets for 30 s in diluted *C. divergens* V41 supernatant, before the cold-smoking process. Two trials were carried out, one in summer, where temperature during the smoking process reached 33 °C, and one in winter with a lower temperature. In the first trial, the bacteriocin treatment achieved a maximum 3-log cycles reduction of *Listeria innocua* viable counts at week 1 of storage, followed by regrowth of the bacterium. In the second trial, a stronger listericidal effect was obtained. No cells of *L. innocua* were found after smoking or at the end of the storage period (Vaz-Velho et al. 2005). These results underline the influence of food processing conditions on the efficacy of bacteriocins, particularly those conditions affecting growth of the target bacterium.

In vacuum-packaged CSS, growth of *L. monocytogenes* could be prevented by a combination of carbon dioxide, nisin, NaCl, and low temperature (Nilsson et al. 1997). Preservation of vacuum packed CSS stored at 5 °C with nisin (500 or 1,000 IU/g) initially reduced the cell numbers of *L. monocytogenes* but did not further prevent growth of survivors (Nilsson et al. 1997), a behavior typically observed when bacteriocins are used at sub-inhibitory concentrations. For that reason, nisin was tested in combination with other antimicrobials, including modified atmosphere packaging, in order to improve control of this pathogen. In experiments carried out in a culture broth, the antilisterial effect of nisin was improved in the presence of 100 % CO_2 and increasing NaCl concentrations (0.5–5.0 % w/v). In CSS packed under MAP (70 %/30 % CO_2/N_2), addition of 500 and 1,000 IU nisin/g inhibited growth of *L. monocytogenes* considerably, with lag phases of 8 and 20 days, respectively, and the levels of *L. monocytogenes* remained below 10 CFU/g during 27 days of storage at both concentrations of nisin (Nilsson et al. 1997).

On smoked salmon slices inoculated with *L. monocytogenes* and surface-treated with nisin (400 or 1,250 IU/g) or ALTA™ 2341 (1 %), both antimicrobials reduced the growth of listeria to some extent (Szabo and Cahill 1999). When the treated smoked salmon was packaged in 100 % CO_2, counts of *L. monocytogenes* were reduced below detectable levels (2 logs) in both cases during 21 days of storage at 4 °C. Addition of these bacteriocins also showed a potential preservation and safety advantage if product was exposed to short-term temperature abuse (Szabo and Cahill 1999).

Chemical preservatives potassium sorbate, sodium lactate and sodium diacetate have strong inhibitory effects on growth of *L. monocytogenes* in CSS. For smoked salmon fillets, the most effective treatment was 2.4 % sodium lactate/0.125 % sodium diatetate, which was able to inhibit the growth of *L. monocytogenes* in smoked salmon fillets for 4 weeks of storage at 4 °C. Nisin showed greatest inhibitory effects in combination with potassium sorbate (0.00125 % nisin/0.15 % potassium sorbate), being able to inhibit the growth of *L. monocytogenes* to levels below 4 log CFU/g for 3 weeks (Neetoo et al. 2008b). Addition of nisin or sodium lactate also inhibited the growth of *L. monocytogenes* in cold-smoked rainbow trout, but the combination of the two compounds was more effective (Nykanen et al. 2000). Nisin, sodium lactate or their combination were injected into rainbow trout at an industrial scale before the smoking process, or injected into the finished smoked product. Best results were obtained when the combination of nisin and sodium lactate (120–180 IU nisin/g + 18 g lactate/ kg) were injected into the smoked fish, decreasing the count of *L. monocytogenes* from 3.26 to 1.8 log CFU /g over 16 days of storage at 8 °C. In the fish injected before smoking, the combination of 3.6 % sodium lactate and 240–360 IU/g nisin or 1.8 % sodium lactate and 120–180 IU/g nisin inhibited growth of *L. monocytogenes* (to almost constant levels of 4.7–4.9 log CFU/g) for 29 days at 3 °C in the vacuum-packed cold smoked samples (Nykanen et al. 2000). In both cases (before or after smoking), application of the combined antimicrobial treatments did also reduce mesophilic aerobic counts in the cold-smoked product. The treatments did not affect the sensory characteristics.

The efficacy of bacteriocins in seafoods can improve with immobilisation in packaging materials. Packaging CSS in plastic film coated with bacteriocin from *Lactobacillus curvatus* CWBI-B28 caused *L. monocytogenes* inactivation late during refrigeration storage (Ghalfi et al. 2006). However, best results (complete inactivation of *L. monocytogenes* during storage for 22 days) were reported for CSS treated with bacteriocin adsorbed to its heat-inactivated producer cells. In CSS vacuum-packed inside nisin-coated plastic films, nisin (2,000 IU/cm^2) inhibited the proliferation of a cocktail of *L. monocytogenes* strains (Neetoo et al. 2008a). Viable counts were 3.9 log CFU/cm^2 lower compared with controls for samples inoculated with 5×10^2 CFU/cm^2 of *L. monocytogenes* after 56 days of storage at 4 °C or 49 days at 10 °C. In addition, nisin inhibited the proliferation of background microbiota (aerobic, anaerobic, and LAB counts) on smoked salmon at both storage temperatures although the bacteriostatic effect was much more pronounced at 4 °C (Neetoo et al. 2008a). Chitosan dosed with 500 IU/cm^2 nisin slowed down growth of *L. monocytogenes* on CSS for 10 days at room temperature by approximately 1 log unit (Ye et al. 2008). Chitosan-coated plastic films dosed with sodium lactate (2.3 mg/cm^2) in com-

bination with nisin (500 IU/cm^2) inhibited the proliferation of *L. monocytogenes* in the vacuum-packaged CSS during storage at 4 °C for up to 6 weeks (Ye et al. 2008). The addition of nisin allowed a reduction in the concentration of sodium lactate from 4.5 mg/cm^2 (when tested singly) to 2.3 mg/cm^2 in the combination while achieving a similar antilisterial effect at least during the first 4 weeks of storage.

In another study, nisin and lysozyme (from hen egg and from oysters) were tested on CSS stored at 4 °C (Datta et al. 2008). The combinations of the two antimicrobials were applied directly or in calcium alginate coating. The effectiveness of oyster lysozyme or hen egg white lysozyme was enhanced when added incorporated onto calcium alginate coatings. After 35 days at 4 °C the growth of *L. monocytogenes* and *Salmonella* Anatum was suppressed in the range of 2.2–2.8 log CFU/g with nisin-lysozyme (from oysters as well as from hen egg) calcium alginate coatings compared to the control nontreated samples, with no significant differences from the source of lysozyme. The study concluded that calcium alginate coatings containing oyster lysozyme and nisin could be used to control the growth of *L. monocytogenes* and *Salmonella* Anatum on the surface of ready-to-eat smoked salmon at refrigerated temperatures.

Bacteriocins have been tested also in other ready-to-eat seafood products with the purpose of enhancing shelf life or reducing the risk of *L. monocytogenes*. In smoked salmon paté, results reported for nisin were not satisfactory compared to organic acid salts of potassium sorbate and sodium lactate or sodium diacetate (Neetoo et al. 2008b). Pâté samples supplemented with organic acid salt treatments had lower counts by the end of 3 weeks compared to those incorporating nisin or nisin with organic acid salts at a lower concentration. It was suggested that the ingredients in pâté provided nisin protection for *L. monocytogenes* (Neetoo et al. 2008b). In fish spreads made from hake flesh, enterocins 1071A and 1071B inhibited the growth of aerobic mesophilic bacteria during cold storage (Dicks et al. 2006). An enterocin concentrate obtained by ammonium sulphate precipitation was added to the fish spreads at 1.0 % (w/w), equivalent to 1.2×10^5 AU/g fish spread. The number of microbial cells recorded in fish spread preserved with enterocins was 8×10^6 CFU/g after 21 days of cold storage (4 °C), compared to 1×10^8 CFU/g in fish spread that had not been preserved. Enterocins 1071A and 1071B did preserve the fish spread, but to a lesser extent than a combination of sodium benzoate and potassium sorbate.

Cooked shelled crabmeat is prone to cross contamination with raw product by personnel and from raw crab in the processing environment. Washing crabmeat with antimicrobials (PerLac 1911, Microgard™, Alta™ 2341, nisin, or *Enterococcus faecium* 1083 culture supernatant containing the bacteriocin-like substance (BLIS) enterocin 1083 reduced the viable counts of *L. monocytogenes* during storage at 4 °C only in the samples treated with 20,000 AU of Alta™ 2341, nisin, or enterocin 1083 (Degnan et al. 1994). However, best results were reported for sodium diacetate or trisodium phosphate. While trisodium phosphate considerably increased the pH of crabmeat to unacceptable levels, sodium diacetate did not produce adverse effects and reduced the levels of *L. monocytogenes* by 2.6 log units/g within 6 day.

Shucked lobster meat is prepared from lobsters cooked by immersion in boiling water, cooled, and shucked by hand. The meat portions are packed with brine and

eaten without further cooking. The risks of bacterial contamination during processing and the zero tolerance level imposed by administrations for *L. monocytogenes* in ready-to-eat seafood products strengthen the needs to apply additional preservation methods. In one study, the combined effect of nisin and moderate heat on *L. monocytogenes* in cans of cold-pack lobster was investigated (Budu-Amoako et al. 1999). Heat processing lobster meat in the presence of nisin (25 mg/kg of can content) at 60 °C internal temperature for 5 min achieved 3- to 5-log reductions in *L. monocytogenes* viable cell counts, whereas nisin or heat alone achieved 1- to 3-log reductions. The effect of the combined treatment was considered to be satisfactory, since reported *L. monocytogenes* levels in the commercial product never exceeded 10^2 CFU/g. An additional advantage was that a reduced heat process in combination with nisin allowed a considerable reduction in drained weight loss compared to the standard heating process used by industry.

In brined shrimp, addition of carnocin UI49 (from *C. piscicola* UI49) did not extend the shelf life, while crude bavaricin A (a cell-free supernatant of *Lactobacillus bavaricus* MI 401) resulted in a shelf life of 16 days, while a nisin Z preparation extended the shelf life for 31 days (Einarsson and Lauzon 1995). The addition of nisin Z and bavaricin A preparations extended the product shelf-life, although the efficacy of bacteriocin treatments was much more limited compared to brined shrimp stored in a benzoate-sorbate solution (Einarsson and Lauzon 1995). It was observed that the dominant microbiota towards the end of treatments was dominated by Gram-positive bacteria in samples treated with carnocin UI49 and bavaricin A as well as in the untreated controls, while in the nisin Z treatment a Gram-negative microbiota was more pronounced. In another study, it was shown that dipping in organic acids solutions followed by vacuum packaging and chilled storage can help reduce *L. monocytogenes* and native microbiota, but not *Salmonella*, on fresh shrimps (Wan Norhana et al. 2012). In that study, beheaded and peeled fresh shrimps dipped in solutions containing nisin (500 IU/ml), EDTA (0.02 M), potassium sorbate (PS) (3 %, w/v), sodium benzoate (SB) (3 %, w/v) or sodium diacetate (SD) (3 %, w/v) alone or in combination were vacuum packaged and stored at 4 °C for 7 days. Nisin-EDTA-potassium sorbate and nisin-EDTA-sodium diacetate significantly reduced *L. monocytogenes* numbers, but none of treatments reduced *Salmonella* counts on shrimps throughout storage. Overall, the applied treatments improved the microbiological quality of shrimps. For example, on day 7 for storage, numbers of aerobic bacteria, psychrotrophic bacteria and *Pseudomonas* on combined nisin-EDTA-salt of organic acids treated shrimps were significantly lower by 4.40–4.60, 3.50–4.01, and 3.84–3.99 log CFU/g respectively, as compared to the control (Wan Norhana et al. 2012).

Caviar is heat labile, and conventional pasteurization processes affect its texture, color, and flavor negatively. Refrigerated storage is currently the only available means to preserve and extend the shelf life of caviar as a ready-to-eat product. Chum salmon caviar (ikura) and sturgeon caviar were treated by immersion in 500 IU/ml nisin solution and heat processed (an 8-D process without nisin or a 4-D process with 500 IU/ml nisin) in a radio frequency (RF; 27 MHz) heating method at 60, 63, and 65 °C (Al-Holy et al. 2004). The combination of RF heating and nisin

acted synergistically to inactivate *L. innocua* cells and total mesophilic microorganisms. No surviving *L. innocua* were recovered in the caviars after application of the nisin-RF combined treatments at 65 °C. The come-up times in the RF-heated product were significantly lower compared with the water bath–heated caviar at all treatment temperatures. The visual quality of the caviar products treated by RF with or without nisin was comparable to the untreated control. The effect of nisin, chemical antimicrobials or moderate heat (singly or in combination) on inactivation of *L. monocytogenes* in sturgeon caviar was further investigated. Treating caviar with 500 or 750 IU/ml nisin initially reduced *L. monocytogenes* by 2–2.5 log units (Al-Holy et al. 2005). Nisin in combination with 2 % lactic acid plus 134 ppm chlorous acid reduced viable counts of *L. monocytogenes* below detectable levels at several points during storage at 4 °C, and it also reduced total mesophilic viable counts. However, best results were obtained for the combinations of nisin and mild heat (60 °C for 3 min). Mild heating in combination with nisin synergistically reduced viable counts of *L. monocytogenes* and total mesophiles. No *L. monocytogenes* cells were recovered from caviar treated with heat and nisin (750 IU/ml) after a storage period of 28 days at 4 °C.

L. monocytogenes can be highly prevalent in minced tuna and fish (salmon and cod) roe, where it can multiply more rapidly under temperature-abuse conditions (Takahashi et al. 2011). Such seafood products are among the most popular sushi ingredients for consumers of all age groups. Since the complete elimination of *L. monocytogenes* from the processing environments in which minced tuna and fish roe products are prepared considered to be very difficult, it is necessary to apply other preservation methods that, at the same time, do not negatively affect the taste of these seafood products (Takahashi et al. 2011). So in one study, nisin in the form of Nisaplin and other antimicrobials (lysozyme, e-polylysine, and chitosan) were tested for inhibition of *L. monocytogenes* in the seafoods stored at 10 °C (Takahashi et al. 2011). Nisaplin effectively inhibited *L. monocytogenes* growth in minced tuna at 500 ppm and in salmon roe at 250 ppm within their standard shelf lives. Consequently, 500 ppm of Nisaplin, (which is the legal standard for cheese and meat products in many countries), was considered an appropriate and safe concentration for seafood.

Karashi-mentaiko is red-pepper seasoned cod roe. However, *L. monocytogenes* has been isolated from Karashi-mentaiko, and since there is no heat treatment in the manufacturing process of Karashi-mentaiko, the control of bacteria is very important (Hara et al. 2009). Nisin can effectively inhibit growth of *L. monocytogenes* in Karashi-mentaiko (Hiwaki et al. 2007). In tests carried out independently on eight different strains, the number of *L. monocytogenes* in Karashi-mentaiko stored at 4 °C was decreased by Nisaplin added at 60 and 600 μg/g (Hara et al. 2009). In the samples containing 60 μg/g Nisaplin, most of the isolates were undetected (except for two strains) through the whole storage period (28 days), while at 600 μg/g Nisaplin none of the strains were detected. When samples were stored at 15 °C, both concentrations of Nisaplin (60 and 600 μg/g) brought all strains undetectable during the whole storage period. Interestingly, the MICs for Nisaplin obtained in Karashi-mentaiko were lower compared to BHI broth, suggesting that ingredients of Karashi-mentaiko, storage temperature and a_w influenced the efficacy of Nisaplin.

7.2 Application of Bacteriocin-Producing Strains

Bacteriocin production by fish-acclimatized bacterial species is of great interest for inhibition of pathogenic microorganisms in seafood products (Table 7.1). Antagonistic bacterial strains (such as those isolated from cold smoked seafood products) could be applied for the competitive exclusion of *L. monocytogenes* in the processed food products. Many LAB strains are able to grow at refrigeration temperatures. They tolerate modified atmosphere packaging, low pH, high salt concentrations, and the presence of additives such as lactic acid, ethanol, or acetic acid. The selected antagonistic strains should meet several criteria: (1) to be able to grow on the fish product during cold storage and produce antimicrobials to inactivate *L. monocytogenes*, or at least inhibit growth of the pathogen; (2) do not cause adverse effects on the food product (such as off flavours, colour changes); (3) do not have adverse effects on health (e.g., production of biogenic amines) or carry antibiotic resistance or virulence traits. Inoculated strains could have probiotic properties, but this approach for administration of probiotics through seafood products has not been exploited yet.

Since LAB comprise the dominant microbiota in CSS (González-Rodríguez et al. 2002; Cardinal et al. 2004), research has focused on selection of antagonistic LAB strains from the processed products. *L. monocytogenes* can be inhibited by carnobacteria cultures that do not produce bacteriocins, partly due to glucose depletion (Nilsson et al. 2005). However, LAB strains producing bacteriocins (mainly *Carnobacterium* and *Lactobacillus* species) may be superior for biopreservation compared to non-bacteriocinogenic strains. Examples of trials carried out with antagonistic bacteria in CS foods (such as CSS, cold-smoked salmon-trout, or cold-smoked surubim) included antagonistic strains of *C. piscicola*, *C. divergens*, *Lactobacillus sakei*, *Lactobacillus casei*, *Lactobacillus curvatus*, *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, *Pediococcus acidilactici* or *E. faecium* (Leisner et al. 2007; Calo-Mata et al. 2008; Galvez et al. 2008; Rihakova et al. 2009; Tomé et al. 2008; Tahiri et al. 2009b).

C. piscicola is often isolated as the naturally dominant LAB species on CSS (Paludan-Müller et al. 1998). Therefore, CSS may be a good source for isolation of bacteriocin-producing LAB. The strains *C. piscicola* A9b (producer of carnobacteriocin B2) and *C. piscicola* CS526 (producer of piscicolin CS526) showed anti-Listeria activity in salmon juice and in CSS, respectively (Nilsson et al. 2004; Yamazaki et al. 2003). Also, the strains *C. divergens* V41 and *C. piscicola* V1 from processed seafoods were reported to be highly effective against *L. monocytogenes* in co-culture experiments carried out in a simulated cold smoked fish system at 4 °C (Duffes et al. 1999b). In cold-smoked surubim (a native Brazilian freshwater fish), inhibition of *L. monocytogenes* by the bacteriocinogenic strain *C. piscicola* C2 isolated from vacuum-packed cold-smoked surubim and by other *C. piscicola* strains isolated from CSS was reported (Alves et al. 2005). Strong inhibition was detected both in fish peptone model systems and in cold-smoked fish juices. Although the carnobacteria grew poorly on cold-smoked surubim at 10 °C, the strains were able

to reduce maximum *Listeria* counts by 1–3 log units in an artificially inoculated surubim. In another study, when an "alecrim pimenta" extract was tested in combination with strains of *Carnobacterium maltaromaticum* (bacteriocinogenic or not), variable effects were observed on *L. monocytogenes* (dos Reis et al. 2011), ranging from strong inhibition to only transient inhibition, depending on the substrate (surubin broth, or surubin homogenate). It was concluded that the use of alecrim pimento extract and cultures of carnobacteria have potential to inhibit *L. monocytogenes* in fish systems and the applications should be carefully studied, considering the influence of food matrix.

Experimental work carried out with bacteriocin-producing lactobacilli indicates that these bacteria can inhibit *L. monocytogenes* in seafood products. Cultures of *L. sakei* L6790 (producer of sakacin P) inoculated on CSS only had a bacteriostatic effect on *L. monocytogenes*, similarly to an isogenic (bac$^-$) *L. sakei* strain. However, application of the bacteriocinogenic culture in combination with a sub-lethal concentration of purified sakacin P resulted in a partial inactivation of *L. monocytogenes* population (Katla et al. 2001). In another study, the bacteriocinogenic strain *L. curvatus* CWBI-B28 was tested against *L. monocytogenes* in CSS during storage at 4 °C by using different approaches: producing bacteriocin *in situ*, spraying with partially purified bacteriocin, packaging in bacteriocin-coated plastic film, and cell-adsorbed bacteriocin (a suspension of producer cells on which maximum bacteriocin has been immobilized by pH adjustments) (Ghalfi et al. 2006). In spite of the fact that all different treatments achieved some inactivation of *L. monocytogenes* in CSS, the cell-adsorbed bacteriocin provided best results, with complete inactivation of listeria for up to 20 days (Ghalfi et al. 2006). Vescovo et al. (2006) evaluated the biopreservative potential of three antimicrobial-producing LAB strains (*L. casei*, *L. plantarum* and *C. piscicola*) on refrigerated CSS stored under vacuum. All three strains were able to inhibit growth of *L. innocua* and none affected negatively the sensory quality of the product. The combination of *L. casei-L. plantarum* was the most effective in inhibition of *Listeria*, while a *L. casei–C. piscicola* association was less effective than *C. piscicola* alone (Vescovo et al. 2006). In another study, bacteriocin-producing LAB (*E. faecium* ET05, *L. curvatus* ET06, *L. curvatus* ET30, *L. delbrueckii* ET32 and *P. acidilactici* ET34), selected by their capacity for growth and producing inhibition in vitro under conditions simulating cold-smoked fish (at high salt-on-water content, low temperature and anaerobic atmosphere) were inoculated onto salmon fillets in co-culture with *L. innocua* 2030c, and cold-smoked processed. The finished product was then packed under vacuum and stored at 5 °C (Tomé et al. 2008). *L. curvatus* ET30 and *L. delbrueckii* ET32 showed a good biopreservation potential for CSS, while *L. curvatus* ET06 and *P. acidilactici* ET34 showed a bacteriostatic mode of action against the target bacteria in vitro as well as when inoculated into the salmon fillets. Comparatively, strain *E. faecium* ET05 showed the best results in controlling *L. innocua* growth in vacuum-packaged CSS processed under the salting/drying/smoking.

Bioprotective cultures can also be applied during the dry-salting process. For example, commercial preparations of *P. acidilactici* (Fargo-35, Laboratorios Amerex S.A., Madrid, Spain), *L. curvatus* (InhiList-2, Innaves S.A., Pontevedra,

Spain), and a mixture of lactic cultures and species extract (Biamex-01, Laboratorios Amerex S.A., Madrid, Spain) were tested on fresh salmon in combination with a dry salt-sugar mix and stored at 8 °C (Montiel et al. 2013). All antimicrobials tested were effective in inhibiting growth of *L. monocytogenes*. After 7 days of storage, the biopreservative based on *P. acidilactici* strongly inhibited growth of the pathogen, with counts 3.6 and 1.5 log CFU/g lower than in the control and salt-sugar mix-treated samples, respectively (Montiel et al. 2013).

Research in enterococci from seafoods has gained interest for biocontrol of *L. monocytogenes* in the processed products, and bacteriocin-producing strains have been isolated from seafoods such as *E. faecium* and *Enterococcus mundtii* strains producers of unknown bacteriocins (Campos et al. 2006; Hosseini et al. 2009; Valenzuela et al. 2010), or *E. faecium* strains producers of enterocin P (Arlindo et al. 2006) or enterocin B (Pinto et al. 2009). *Enterococcus* isolates from sea bass and sea bream showed broad antimicrobial activities (against *Carnobacterium* sp., *Bacillus* sp., *L. monocytogenes*, *Aeromonas salmonicida*, *Aeromonas hydrophila* and *Vibrio anguillarum*) and carried enterocin genes (including enterocins A, B, L50 and P), strengthening the potential applications of these LAB strains to the biopreservation of minimally-processed seafood products (Chahad et al. 2012).

Also, enterococci from other sources have been suggested for application in seafoods. An *E. mundtii* strain isolated from soil with strong anti-*Listeria* activity was tested in vacuum-packed CSS stored at 5 °C (Bigwood et al. 2012). This strain inhibited the growth of *L. monocytogenes* on the CSS during its 4 week shelf life. When *L. monocytogenes* (ca. 3.4 log CFU/cm^2) was co-inoculated with *E. mundtii* (7 log CFU/cm^2), growth of the pathogen was reduced compared to the control samples with an approximate 3 log CFU/cm^2 difference in concentration after 4 weeks incubation (Bigwood et al. 2012). The inhibitory effect was found to be dependent on the initial inoculum of enterococci. The *E. mundtii* isolate was able to grow at 5 °C in culture medium, but not on the CSS, and for that reason a high inoculum of enterococci was required in order to achieve a strong inhibition of the listeriae. The study concluded that *E. mundtii* could control the growth of *L. monocytogenes* at low temperatures, indicating a potential application in controlling this pathogen in chilled foods.

While most studies have focused on inhibition of *L. monocytogenes* in seafood products, other pathogenic bacteria (such as *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio cholerae*, *Clostridium botulinum*, histamine-producing bacteria, and post-contaminating bacteria, such as *S. aureus* or *Salmonella* sp.) or spoilage bacteria (such as *Shewanella putrefaciens*, *Photobacterium phosphoreum*, *Aeromonas* spp. and *Pseudomonas* spp.) are still a matter of concern (Gram and Dalgaard 2002; Calo-Mata et al. 2008). Therefore, there is a growing interest to extend the spectrum of inhibition of bacteriocins in combination with other hurdles and also on isolation of LAB strains with broader spectrum of inhibitory activity. One study reported that treatment of whole shrimp with potassium sorbate in combination with *Bifidobacterium breve* cells extended the product microbiological shelf life (Al-Dagal and Bazaraa 1999). Also, treatment of plaice fillets with a preparation of *Bifidobacterium bifidum* cells and thymol combined with low storage temperature and anoxia/hypoxia, showed a great efficacy against the main fresh

packaged fish spoilage species (Altieri et al. 2005). Selected strains of *Leuconostoc gelidum*, *Lactococcus piscium*, *Lactobacillus fuchuensis*, and *Carnobacterium alterfunditum* (psychrotrophs, lacking antibiotic resistance traits and unable to produce histamine or tyramine) have been investigated as broad-spectrum bio-protective cultures in fish preservation (Matamoros et al. 2009).

7.3 Bacteriocin Cultures in Fermented Fish

Fermented fish products are very popular in the Asiatic and Pacific regions, but their microbiological aspects are not known so well as other fermented foods. Salted fermented foods contain abundant amino acids, which can generate relatively large amounts of biogenic amines (Mah et al. 2003). Inoculation with *Staphylococcus xylosus* producer of a BLIS has been proposed as a protective culture to decrease biogenic amine formation in salted and fermented anchovy (Mah and Hwang 2009).

L. lactis subsp. *lactis* strain CWBI B1410 (which produces various antibacterial compounds including organic acids and nisin) was tested to improve the traditional Senegalese fish fermentation into guedj (Diop et al. 2009). The inoculated starter (in combination with glucose addition) released nisin onto the fish fermentate, produced a faster acidification and reduced the counts of enteric bacteria in the fermented fish. The authors proposed a new fish fermentation strategy based on inoculation with this strain as starter, combined with salting and drying to enhance the safety of guedj.

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Chapter 8
Biopreservation of Vegetable Foods

8.1 Application of Bacteriocins

8.1.1 Fresh Produce

Fresh produce products can become contaminated with human pathogenic bacteria from different sources (such as manure, irrigation water, insects, and during harvesting and other process operations), and have been implicated in a number of outbreaks (Lynch et al. 2009). Several bacteriocin preparations (such as nisin, pediocin, or enterocin AS-48) have been assayed for inactivation of foodborne pathogenic or toxinogenic bacteria (such as *Listeria monocytogenes*, *Bacillus cereus*, and *Bacillus weihenstephanensis*, *Escherichia coli*, *Salmonella* and other enterobacteria) on the surfaces of fresh-cut vegetables and on sprouted seeds (Galvez et al. 2008; Randazzo et al. 2009; Abriouel et al. 2010) (Table 8.1). Bacteriocin treatments have also been proposed for decontamination of whole fruit surfaces, and to avoid transmission of pathogenic bacteria from fruit surfaces to processed fruits (Ukuku et al. 2005; Silveira et al. 2008), and to decrease bacterial survival of bacteria on sliced fruit surfaces during storage.

Several studies have reported inactivation of human pathogenic bacteria in salads. Torriani et al. (1997) reported that salad vegetables treated with culture supernatant of a *Lactobacillus casei* strain reduced the coliform count, but the presence of a bacteriocin was not confirmed. Randazzo et al. (2009) tested the effect of bacteriocin RUC9 from a wild strain of *Lactococcus lactis* (previously isolated from minimally processed mixed salads) in minimally processed iceberg lettuce samples artificially inoculated with a wild strain of *L. monocytogenes* during storage at 4 °C, in comparison with commercial nisin. None of the bacteriocin treatments completely eliminate the pathogen on the produce, but RUC9 treatment achieved a greater reduction of *L. monocytogenes* viable counts after 7 days of storage at 4 °C compared to nisin (2.7 log units versus 1 log unit). It was suggested that treatment with RUC9 bacteriocin could be used as sanitizer to improve microbial safety and

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Table 8.1 Example applications of bacteriocin and bacteriocin-producing LAB in vegetable foods and beverages

Bacteriocin treatment	Effect(s)	Reference(s)
Nisin Z, coagulin, nisin:coagulin cocktail	Reduced viable cell counts of *L. monocytogenes* on fresh-cut iceberg lettuce stored in microperforated plastic bags	Allende et al. 2007
Enterocin AS48 washing treatments alone or in combination with other antimicrobials	Inactivation of *L. monocytogenes, B. cereus, B. weihenstephanensis* and *Enterobacteria* on sprouts	Abriouel et al. 2010
Nisin in combination with of hydrogen peroxide, sodium lactate and citric acid as a sanitizer	Decontamination of whole cantaloupe and honeydew melon surfaces. Prevented transfer of *L. monocytogenes* and *E. coli* to fresh cut pieces	Ukuku et al. 2005
Nisin combination with lysozyme and pulsed electric fields (PEF)	Inactivation of *Salmonella* Typhimurium in orange juice	Liang et al. 2002
Enterocin AS-48 alone or in combination with PEF, chelators, or heat	Inactivation of pathogenic and spoilage bacteria in fruit juices	Abriouel et al. 2010
Nisin and high pressure homogeneization	Improved inactivation of *L. monocytogenes* in juice	Pathanibul et al. 2009
Nisin and high hydrostatic pressure	Reduction of aerobic mesophlic microbiota in cucumber juice	Zhao et al. 2014
Nisin	Prevented spoilage caused by non-aciduric and aciduric spore formers in canned foods and in other foods	Thomas et al. 2000, 2001
Enterocin AS-48	Inactivation of endospore formers in boiled rice, purees, and canned vegetables	Abriouel et al. 2010
Nisin	Inactivation of wine LAB at lower sulphite concentrations	Rojo-Bezares et al. 2007
Pediocin PD-1	Control of *O. oeni* in wines	Bauer et al. 2003
Enterocins L50A and L50B	Inhibition of beer spoilage LAB in worts and lager beers	Basanta et al. 2008
Plantaricin-producing starter culture	Improved microbiological control of table olives fermentation	Vega Leal-Sánchez et al. 2003
Kimchicin-producing *Leuconostoc citreum*	Inhibition of foodborne pathogens in kimchi	Chang and Chang 2011
LactiGuard™ lactic acid bacteria	Inhibition of *E. coli* O157:H7 and *C. sporogenes* in spinach	Brown et al. 2011
Bacteriocin-producing strains	Inhibition of rope-forming bacilli in breads	Settanni and Corsetti 2008
	Enhanced competitiveness of strains in fermented doughs	
Bacteriocin-producing *L. plantarum*	Decreased survival of *B. cereus*, *E. coli* O157:H7 and *S. enterica* in millet gruels	Sánchez Valenzuela et al. 2008

to reduce the chemical treatment in vegetable processing. Allende et al. (2007) tested bacteriocin preparations (nisin Z, coagulin and a nisin:coagulin cocktail) produced by cultivation of selected LAB strains on a lettuce extract in fresh-cut Iceberg lettuce stored in microperforated plastic bags. The applied bacteriocin extracts reduced viable cell counts of *L. monocytogenes*, but did not prevent further growth of survivors during refrigeration storage of samples.

Nisin and pediocin individually or in combination with sodium lactate, potassium sorbate, phytic acid, and citric acid were tested as possible sanitizer treatments for reducing the population of *L. monocytogenes* on cabbage, broccoli, and mung bean sprouts (Bari et al. 2005). After 1-min wash, the combination treatments nisin-phytic acid and nisin-pediocin-phytic acid caused significant reductions of *L. monocytogenes* on cabbage and broccoli but not on mung bean sprouts. Pediocin treatment alone or in combination with any of the organic acid tested was more effective in reducing *L. monocytogenes* populations than the nisin treatment alone (Bari et al. 2005).

Bennik et al. (1999) applied a solution containing 200 AU/ml of mundticin ATO6 (from *Enterococcus mundtii* ATO6) on mung bean sprouts by dipping or coating with an alginate film. Mundticin treatments reduced the levels of *L. monocytogenes* by ca. 2 log cycles after the treatments. However, the bacteriocin treatment had no effect on growth of the listeriae during storage of the treated sprouts under a modified atmosphere (MA) at 8 °C. In another study, alfalfa and soybean sprouts artificially contaminated with *L. monocytogenes* were treated by immersion for 5 min in solutions containing enterocin AS-48 of 5.0, 12.5 and 25 μg/ml. Best results were obtained for samples treated with 25 μg/ml and stored right after treatments at temperatures of 6 or 15 °C, achieving reductions in viable counts of 2.0–2.4 log cycles (Cobo Molinos et al. 2005). In alfalfa and soybean sprouts, the 25 μg/ml bacteriocin treatment reduced viable counts below detection levels and prevented overgrowth of the listeria for a 7-days storage period at temperatures of 6, 15, and 22 °C. However, the same bacteriocin treatment provided more heterogeneous results in green asparagus and failed to inhibit overgrowth of the listeria during storage. The listeridical effect on green asparagus was strongly potentiated when the bacteriocin was applied in combined treatments (like, for example, with lactic acid, trisodium trimetaphosphate, n-propyl-p-hydroxybenzoate, peracetic acid, or sodium hypochlorite). In sprouts and green asparagus artificially contaminated with *B. cereus* and *B. weihenstephanensis*, washing treatments with enterocin AS-48 (25 μg/ml) reduced viable cell counts by up to 2.4 log cycles in samples stored at 6 °C, but not at 15 or 22 °C (Cobo Molinos et al. 2008a). Microbial inactivation improved when the bacteriocin was tested in combination with other antimicrobials (sodium hypochlorite, peracetic acid, polyphosphoric acid or hydrocinnamic acid). For inactivation of *S. enterica* on sprouts, the most effective treatments consisted of solutions containing enterocin AS-48 (25 μg/ml) with lactic acid or polyphosphoric acid (Cobo Molinos et al. 2008b). The combinations of enterocin AS-48 (25 μg/ml) and polyphosphoric acid in a concentration range of 0.1–2.0 % significantly reduced or inhibited growth of the populations of *S. enterica* as well as other Gram-negative bacteria (*E. coli* O157:H7, *Shigella* spp., *Enterobacter aerogenes*, *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Pseudomonas fluorescens*) in sprout samples stored at 6 and 15 °C.

Fresh-cut fruits have been involved in a number of outbreaks due to cross-contamination with human pathogenic bacteria. Nisin reduced *L. monocytogenes* populations on honeydew melon slices and apple slices (Leverentz et al. 2003). Combination treatments containing nisin-sodium lactate, nisin-potassium sorbate and nisin-sodium lactate-potassium sorbate achieved significant reductions of *Salmonella* directly inoculated onto fresh-cut cantaloupe pieces (Ukuku and Fett 2004). Decontamination of whole cantaloupe and honeydew melon surfaces with a combination of hydrogen peroxide, nisin, sodium lactate and citric acid as a sanitizer prevented further transfer of the inoculated *L. monocytogenes* and *E. coli* to fresh cut pieces (Ukuku et al. 2005). In another study, whole fruit pieces and sliced fruits were decontaminated with an enterocin AS-48 solution (25 μg/ml) (Cobo Molinos et al. 2008c). In the absence of treatments, it was found that *L. monocytogenes* was able to multiply in the less acidic sliced (such as pear, kiwi, melon or watermelon). Bacteriocin treatments significantly inhibited or completely inactivated *L. monocytogenes* in strawberries, raspberries, and blackberries stored at 15 and 22 °C for up to 2 days and in blackberries and strawberries at 6 °C for up to 7 days. Washing treatments also reduced viable counts in sliced melon, watermelon, pear, and kiwi but did not avoid proliferation of survivors during storage at 15 and 22 °C. However, it was reported that combinations of enterocin AS-48 with carvacrol or with *n*-propyl *p*-hydroxybenzoate avoided overgrowth of listeria on sliced melon during storage at 22 °C. Some of the combined treatments proposed could find industrial applications, especially in added-value food products such as those intended for consumption by the elderly, immunocompromised people, or debilitated hospital patients.

8.1.2 Fruit Juices and Drinks

Bacteriocins could be exploited for biopreservation of in fruit and vegetable juices and drinks. These substrates exhibit certain features that may enhance bacteriocin activity and stability, e.g.: (1) improved diffusion of bacteriocin molecules (compared to solid substrates), (2) a low fat content in general, minimizing bacteriocin adsorption to hydrophobic food components, (3) an acidic pH, which in general facilitates bacteriocin solubility and activity and (4), they usually contain organic acids and other bioactive molecules which may potentiate bacteriocin activity. In fruit juices and drinks, bacteriocin addition (nisin, enterocins) has been proposed for inactivation of endospore-forming bacteria causing spoilage such as *Alicyclobacillus acidoterrestris* and thermophilic spore formers such as *Geobacillus stearothermophilus*. Bacteriocin addition may also be useful for microbial inactivation of bacteria causing ropiness (such as exopolysaccharide-producing *Bacillus licheniformis*, pediococci and lactobacilli), as well as acrolein-producing bacteria. While fruit juices and drinks usually have a pH that is too low for proliferation of foodborne pathogenic bacteria, some less acidic juices and drinks can support bacterial growth. Inactivation of foodborne pathogens (*L. monocytogenes*, *B. cereus*,

Staphylococcus aureus) by enterocins has been reported in lettuce juices, soy milk, and sport and energy drinks with lower acidity (Galvez et al. 2008; Abriouel et al. 2010). Since freshly-made fruit juices have been implicated in transmission of enteric pathogens, bacteriocins (such as nisin and enterocin AS-48) have been tested in combination with other agents to increase the bacterial outer membrane permeability. The combined treatments of bacteriocins and PEF greatly increased the bactericidal effects and decreased the risks of survivor proliferation in the treated samples (Liang et al. 2002; Mosqueda-Melgar et al. 2008).

In fruit juices, the thermophilic endospore former *A. acidoterrestris* can withstand pasteurisation temperatures commonly applied during food processing, and spoil freshly-made juices as well as processed juices. Even moderate growth of this bacterium can confer an unpleasant medicinal taste to fruit juices, due to the production of guaiacol. Some bacteriocins (mainly nisin and enterocin AS-48) have been suggested as possible hurdles against this bacterium. Nisin (1.25–100 IU/ml) was able to inhibit *A. acidoterrestris* in orange juice, grapefruit juice and apple juice (Komitopoulou et al. 1999) as well as in orange and fruit-mixed drinks (Yamazaki et al. 2000). Nisin was also able to inhibit spore germination at 25–50 IU/ml in orange and mixed fruit drinks, but not by 600 IU/ml in clear apple juice, probably because of a competitive effect of phenols (Yamazaki et al. 2000). Addition of nisin to orange juice (0, 50, 75, and 100 IU of nisin/ml juice) increased the thermal death of *A. acidoterrestris* spores, with reported decrease in the D value up to 27 % heat resistance as the nisin concentration was increased (Peña et al. 2009). Enterocin AS-48, added at low concentrations of 2.5 μg/ml in fruit juices artificially contaminated with vegetative cells and endospores of *A. acidoterrestris* caused complete bacterial inactivation and afforded protection for up to 14 days in freshly made orange and apple juices and for up to 60–90 days in several commercial fruit juices (Grande et al. 2005). Electron microscopy examination of bacteriocin-treated vegetative cells revealed substantial cell damage and bacterial lysis. Treatment of endospores with enterocin AS-48 caused inhibition of germination and disorganisation of endospore structure.

Another heat-resistant bacterium in fruit juices is the non-sporeformer *Propionibacterium cyclohexanicum*, implicated in the spoilage of orange juice (Kusano et al. 1997). One study showed that addition of nisin (500 and 1,000 IU/ml) to orange juice significantly reduced the viable population of *P. cyclohexanicum* for up to 15 days, but did not prevent regrowth of the bacterium during higher storage periods (Walker and Phillips 2008).

Fruit juices may also be spoiled by bacteria producing exopolysaccharides (EPS), acrolein, or simply by growth causing turbidity. In apple juice and apple ciders, added enterocin AS-48 (2.5–5 μg/ml) was very effective against EPS-producing bacterial strains (including *B. licheniformis* LMG 19409, *Lactobacillus collinoides*, *Lactobacillus diolivorans* and *Pediococcus parvulus*) as well as 3-hydroxypropionaldehyde -producing *L. collinoides* strains (Grande et al. 2006b; Martínez-Viedma et al. 2008a). In coconut juice and coconut milk, addition of enterocin AS-48 (at a final concentration as low as 1.75 μg/ml) completely suppressed *G. stearothermophilus* for at least 30 days of incubation at 45 °C (Martínez-Viedma et al. 2009b).

Many different reports have indicated that unpasteurized fruit juices have been involved in outbreaks due to contamination with pathogenic enteric bacteria such as *E. coli* and *Salmonella enterica*. Since bacteriocins in general are not active on Gram-negative bacteria due to the protective barrier of the bacterial outer membrane, different trials have been carried out in which bacteriocins were tested in combination with outer membrane-permeabilising treatments or antimicrobial agents in order to increase microbial inactivation. In apple juice, a combination of nisin and cinnamon improved the inactivation of *Salmonella* Typhimurium and *E. coli* O157:H7, therefore enhancing the safety of the juice (Yuste and Fung 2004). Nisin (300 IU) in combination with EDTA (20 mM) caused a decline in the populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* in apple cider, suggesting possible addition of this preparation to freshly prepared apple cider to enhance its microbial safety and prevent costly recalls (Ukuku et al. 2009). In apple juice, it was shown that *E. coli* O157:H7 cells sublethally injured by outer membrane permeabilizing treatments (EDTA, sodium tripolyphosphate, pH 5.0, and moderate heat) became sensitive to enterocin AS-48. Highest bactericidal activity (up to to 8.1 log cycles inactivation) was observed when the bacteriocin was applied in multiple treatments (Ananou et al. 2005).

Non-thermal food processing technologies are gaining interest in food preservation. Treatments with high-intensity pulsed electric fields (PEF) are quite effective in microbial inactivation in pumpable substrates such as fruit juices while having little or no effect on organoleptic and nutritional properties (Martín-Belloso and Elez-Martínez 2005; Mittal and Griffiths 2005). Therefore, several studies have explored the synergistic effects of PEF treatments in fruit juices. Addition of nisin (2 %, wt/vol) to freshly squeezed, unpasteurized, and preservative-free apple juice followed by application of a PEF treatment (80 kV/cm, 10 pulses, 42 °C) caused a greater reduction in *E. coli* O157:H7 cell counts of more than 3 log cycles compared to application of PEF treatment alone (Iu et al. 2001).

Nisin and/or lysozyme in combination with PEF treatments were studied on *S.* Typhimurium in pasteurized and freshly squeezed orange juice (Liang et al. 2002). It was found that increasing the treatment temperature to 45 °C or above was critical for inactivation of *Salmonella* by PEF, suggesting a synergistic effect of heat in the inactivation process. Application of PEF treatments (90 kV/cm, 30 pulses, 45 °C) in the presence of nisin (100 U/ml of orange juice), lysozyme (2,400 U/ml), or a mixture of nisin (27.5 U/ml) and lysozyme (690 U/ml), achieved much greater cell inactivation compared with the single treatments. The most effective PEF treatment in juice was obtained using a combination of nisin and lysozyme, achieving a ca. 6.5 log cycle-reduction in viable counts of *Salmonella* (Liang et al. 2002).

Enterocin AS-48 (60 μg/ml) in combination with PEF treatments (35 kV/cm, 1,000 μs) at 40 °C was tested against *S. enterica* cells in apple juice. The combined treatments decreased viable counts of the pathogen by 4.5-log cycles, while treatment with bacteriocin alone had no effect (Martínez-Viedma et al. 2008b). PEF treatment of apple juice combined with enterocin AS-48 (at a subinhibitory concentration of 2 mg/l) also showed higher efficacy against the exopolysaccharide-producing, fruit juice-spoilage strain *L. diolivorans* 29 compared to PEF and

enterocin alone (Martinez-Viedma et al. 2009c). Bacteriocin addition to apple juice also served as an additional hurdle after treatments, preventing regrowth of survivors during storage of treated samples for at least 15 days at 4 and 22 °C (Martinez-Viedma et al. 2009c).

The combinations of bacteriocins with PEF treatments have been explored with the aims to achieve higher reductions of total microbial loads and to prevent or retard food spoilage. In one study carried out on freshly squeezed apple cider, it was shown that inactivation of naturally occurring microorganisms (yeast and molds) increased by 1.1–1.8 logs upon application of PEF treatment in combination with a nisin/lysozyme mixture (Liang et al. 2006). A similar combined treatment was tested for inactivation of naturally occurring spoilage microorganisms in red and white grape juices, achieving reductions in viable bacterial counts up to 5.9 logs (Wu et al. 2005). Also, addition of nisin (100 U/ml) in tomato juice before application of a PEF treatment (80 kV/cm, 20 pulses, 50 °C) was reported to stably reduce cell counts by about 4.4 log units during 28 days of storage at 4 °C, with the advantage that vitamin C content was not affected –as otherwise would happen after application of more intense heat treatments (Nguyen and Mittal 2007).

High pressure homogeneization (HPH) sensitises *E. coli* cells to antibacterial peptides and enzymes (Diels et al. 2004, 2005). In one study, Pathanibul et al. (2009) tested the effect of HPH (0–350 MPa) on *E. coli* and *Listeria innocua* cells in apple or carrot juice. Addition of nisin (10 IU) in combination with HPH increased the bactericidal effect against *L. innocua* in apple juice as well as in carrot juice, reducing to some extent the intensity of the high pressure treatment for inactivation of this bacterium (Pathanibul et al. 2009). However, inactivation of *E. coli* cells in juices did not improve by addition of nisin in combination with HPH. Bacteriocins could also improve the efficacy of high hydrostatic pressure (HHP) treatments in vegetable foods. In one study, it was found that the combination of nisin with HHP had a synergistic effect on the inactivation of total plate count in cucumber juice (Zhao et al. 2014).

8.1.3 Ready-to-Eat, Processed, and Canned Vegetable Foods

Deli-type salads are very popular ready-to-eat vegetable foods. These food products often contain mixtures of cooked and/or uncooked vegetables (e.g. potatoes, tomatoes, olives, peas, carrots, lettuce or cabbage) and other ingredients (e.g. ham, chicken, tuna, egg, or seafood) blended with mayonnaise or salad dressing. Extensive handling during preparation by foodservice personnel, cross contamination, potential abusive temperatures during storage, and the lack of heat-treatment before consumption are factors that may compromise the microbiological safety of these foods. Because of the low numbers of competing microbiota as a result of cooking steps, foodborne pathogens can easily proliferate in salads (Jay et al. 2005). Transmission of bacterial pathogens such as *S. enterica* and *L. monocytogenes* by deli salads is a public health concern (Unicomb et al. 2003; Mokhtari et al. 2006).

Addition of nisin in deli-type salads reduced the concentrations of viable *Listeria* following bacteriocin treatment, but it did not prevent overgrowth of survivors during storage (Schillinger et al. 2001). In vegetable salads (containing a mayonnaise-blended mixture of ingredients such as boiled potato, carrots, peas, olives, egg and tuna) complete inactivation of *L. monocytogenes* population was achieved by adding enterocin AS-48 at 60 μg/g singly or at 30 μg/g in combinations with a variety of antimicrobial substances such as essential oils or their bioactive compounds, and chemical preservatives (Cobo Molinos et al. 2009a). Synergistic effects were also reported in salads against a cocktail of *S. enterica* serovars for AS-48 and *p*-hydroxybenzoic acid methyl esther or 2-nitropropanol (Cobo Molinos et al. 2009b). In vegetable sauces, inactivation of *S. aureus* improved considerably when combinations of enterocin AS-48 and hydrocinnamic acid or carvacrol were added (Grande et al. 2007a).

In cooked vegetable foods, most vegetative cells can be inactivated during the heat processing. However, endospores surviving heat treatments can proliferate in the finished product unless additional hurdles (such as bacteriocins) are included. Enterocin AS-48 added in a concentration range of 20–35 μg/g to boiled rice and in a commercial infant rice-based gruel dissolved in whole milk and inoculated with vegetative cells and endospores of *B. cereus*, completely suppressed the bacilli during storage in a temperature range of 6–37 °C for up to 15 days and prevented enterotoxin production (Grande et al. 2006b). Bacteriocin activity was improved by adding sodium lactate, decreasing the bacteriocin concentration to 8–16 μg/g without compromising inactivation of the bacilli. Application of AS-48 in combination with heat treatments decreased the thermal death *D* values for endospores (Grande et al. 2006b). In desserts and bakery ingredients, the efficacy of AS-48 (added in a concentration range of 5–50 μg/g) against *S. aureus*, *B. cereus*, and *L. monocytogenes* depended to a great extent on the food substrate and the target bacteria (Martínez-Viedma et al. 2009a, b, c). Bacteriocin activity in chocolate cream increased markedly when tested in combination with eugenol, 2-nitropropanol or Nisaplin (Martínez-Viedma et al. 2009c).

L. monocytogenes has been reported to grow on tofu stored at refrigeration temperatures (Schillinger et al. 2001). Nisin showed limited efficacy against *L. monocytogenes* Scott A in tofu. Following an initial reduction of viable counts by nisin, regrowth of survivors was observed during further incubation (Schillinger et al. 2001). To improve microbial inactivation, nisin was tested in combination with bacteriocinogenic strains *Enterococcus faecium* BFE 900-6a or *L. lactis* BFE 902 as protective cultures, resulting in a complete suppression of listerial growth in home-made tofu stored at 10 °C for 1 week (Schillinger et al. 2001).

Canning and cooking processes of vegetables destroy most of the vegetative bacterial forms. Yet, due to the high thermal resistance of endospores and the frequent endospore contamination of raw materials, endospore-forming bacteria represent the main risk for spoilage of foods prepared in this way. Additional hurdles such as refrigeration, acidification, addition of salt or chemical preservatives are often required to avoid proliferation of sporeformers in the processed products. Several studies support the practical application of bacteriocins in this category of

foodstuffs to inhibit endospore outgrowth and also to increase the efficacy of thermal treatments against endospores. Incorporation of nisin in canned vegetables can prevent spoilage caused by non-aciduric (*Bacillus stearothermophilus* and *Clostridium thermosaccharolyticum*) as well as by aciduric (*Clostridium pasteurianum*, *Bacillus macerans*, *Bacillus coagulans*) spore formers (Thomas et al. 2000). Nisin has also been reported to be an effective preservative in fresh pasteurized "home-made"-type soups (Thomas et al. 2000) and in the control of *Bacillus* and *Clostridium* in cooked potato products (Thomas et al. 2002). For example, in pasteurised, vacuum-packaged mashed potatoes inoculated with a cocktail of *Clostridium sporogenes* and *Clostridium tyrobutyricum* spores, addition of nisin prevented bacterial growth and extended the shelf life of the mashed potatoes by at least 30 days (Thomas et al. 2002). Similar results were reported following nisin addition in trials involving a cocktail of *B. cereus* and *Bacillus subtilis* strains (Thomas et al. 2002). Nisin could be applied singly or in combination with other bacteriocins, as shown in sous vide mushrooms, in which addition of a nisin-pediocin mixture prevented outgrowth of *B. subtilis* spores (Cabo et al. 2009).

In cooked vegetables (such as cooked potato products, sous-vide mushrooms, "home-made"-type soups, purees, or cooked rice foods) and in canned vegetables (such as canned tomato, peas, corn, etc.), addition of bacteriocins (such as nisin, nisin-pediocin combination, or enterocin AS-48) has been proposed as a way to inhibit endospore outgrowth and production of enterotoxins (such as *B. cereus* or *Clostridium botulinum* toxins) during storage and/or to increase the efficacy of thermal treatments against endospores (Thomas et al. 2000; Galvez et al. 2008; Cabo et al. 2009; Abriouel et al. 2010). Incorporation of bacteriocins in canned vegetables can be an effective hurdle to prevent spoilage caused by non-aciduric as well as by aciduric spore formers.

In vegetable food products processed by heat such as purees and canned vegetables, enterocin AS-48 was tested against endospore-forming bacteria. In one study, by adding AS-48 (10 mg/l), *B. cereus* LWL1 was completely inhibited in six vegetable products (natural vegetable cream, asparagus cream, traditional soup, homemade-style traditional soup, vegetable soup, and vichyssoise) for up to 30 days in samples stored at 6, 15, and 22 °C. Other *Bacillus* and *Paenibacillus* species and strains isolated from purées showed variable degrees of inactivation by enterocin AS-48, requiring bacteriocin concentrations up to 50 μg /ml (Grande et al. 2007b). Antimicrobial activity on a cocktail of bacilli increased considerably in combination with phenolic compounds (carvacrol, eugenol, geraniol, and hydrocinnamic acid) (Grande et al. 2007b). In canned foods such as tomato paste, syrup from canned peaches, and juice from canned pineapple, *B. coagulans* (responsible for flat sour spoilage) was inhibited by added enterocin AS-48 (6 mg /l) for at least 15 days of storage at 37 °C (Lucas et al. 2006). Added bacteriocin also increased heat inactivation of *B. coagulans* endospores.

In canned corn and peas, addition of enterocin AS-48 (7 μg/g) inactivated *G. stearothermophilus* cells for at least 30 days at a temperature of 45 °C simulating tropical conditions (Martínez Viedma et al. 2010a). Remarkably, AS-48 strongly adsorbed to bacterial endospores, inhibiting endospore outgrowth. Enterocin EJ97

(2–4 AU/ml) could also inactivate *G. stearothermophilus* vegetative cells in canned corn and peas, and it increased the efficacy of heat treatments on endospores (Martínez Viedma et al. 2010a). Enterocin EJ97 immobilized by coating in polythene films in combination with EDTA reduced the concentrations of viable *B. coagulans* cells canned corn and peas stored at 4 °C (Martínez Viedma et al. 2010b).

8.1.4 Fermented Vegetables and Beverages

Most vegetable fermentations are spontaneous (that is, no starter cultures being added) and rely on the selective growth of the microbiota present in the raw materials as well as microorganisms acquired during handluing a processing, coming from water, equipments, and the processing environment. Addition of bacteriocins (such as nisin) has been proposed as a way to direct the microbiota of vegetable fermentations towards selection of desirable bacteriocin-tolerant or bacteriocin-resistant strains with desirable effects (which in part may be due which due to their homofermentative or heterofermentative traits) while at the same time inhibiting strains causing defects such as overripening (as in the case of kimchi fermentation) or spoilage.

Addition of a nisin preparation to cabbage inoculated with nisin-resistant *Ln. mesenteroides* improved control of the fermentation and delayed growth of the homofermentative LAB (Breidt et al. 1995). In kimchi, nisin was added to control lactobacilli responsible for over-ripening of the product. Nisin addition showed higher growth inhibition of *Lactobacillus* spp. than *Leuconostoc* spp. (Choi and Park 2000).

Ropiness of bread is mainly caused by *B. subtilis*, but *B. licheniformis*, *Bacillus megaterium* and *B. cereus* may also be involved. Rope formation may occur in wheat breads that have not been acidified, or in breads with high concentrations of sugar, fat, or fruits (Beuchat 1997). The application of Nisaplin or nisin-producing lactic acid bacteria in bread production was considered to be ineffective for inhibition of *B. subtilis* and *B. licheniformis* strains (Rosenquist and Hansen 1998). The fermented broth from a *Lactobacillus plantarum* strain producing bacteriocin-like inhibitors was reported to inhibit rope formation by *B. subtilis* in yeast-leavened bread (Valerio et al. 2008). Other bakery products may combine a variety of ingredients (as is the case of refrigerated pizza), making the control of microbial spoilage more difficult. In ham pizza, application of Nisaplin under modified atmosphere packaging significantly increased the product shelf life, due to inhibition of spoilage lactic acid bacteria (Cabo et al. 2001).

In fermented beverages, bacteriocin preparations can be applied against spoilage LAB. In the beer production process, several applications have been proposed for nisin (Ogden and Tubb 1985; Ogden and Waites 1986; Radler 1990; Delves-Broughton et al. 1996; Jespersen and Jakobsen 1996; Thomas et al. 2000): (1) cleaning of the equipment and final cleansing rinse; (2) addition to fermenters to control contamination; (3) increasing the shelf life of uncontaminated beers; (4) reduction of pasteurization regimes, and (5) washing pitching yeasts to eliminate

contaminating bacteria, and (6) development of wort bioacidifiying-LAB and/or yeast starter cultures genetically modified to produce bacteriocins. Although nisin activity is limited to Gram-positive bacteria, the sensitivity of the Gram-negative bacterium *Pectinatus frisingensis* to low nisin concentrations was reported in one study (Chihib et al. 1999a). However, selection of a strain resistant to high nisin concentration was also described (Chihib et al. 1999b). Nisin was reported to act synergistically in combination with potassium metabisulphite against wine LAB, and it was proposed that the addition of nisin could be applied in order to reduce the concentrations of sulphur dioxide currently used in the wine industry (Bartowsky 2009; Rojo-Bezares et al. 2007).

Bacteriocins from strains of *Lactobacillus sakei* and *Ln. mesenteroides* isolated from malted barley have been proposed as biological control agents in the brewing industry (Vaughan et al. 2001). Bacteriocins produced by bacterial strains from other sources could also be useful, provided they inhibit target spoilage bacteria in the brewing process. For example, application of thermophilin 110 from *Streptococcus thermophilus* in the brewing industry has been suggested based on its high antimicrobial activity on pediococci (Gilbreth and Somkuti 2005). Enterocins have also been tested for biopreservation of beers. Enterocins L50A and L50B produced by strain *E. faecium* L50 were bactericidal against the most relevant beers spoilage LAB (i.e., *Lactobacillus brevis* and *Pediococcus damnosus*) in a dose- and substrate-dependent manner when challenged in wort, alcoholic and non-alcoholic lager beers at 32 °C. Enterocin addition achieved log reductions of ca 5 log cycles, and no bacterial resistances were detected even after incubation for 6–15 days (Basanta et al. 2008).

Nisin addition is permitted in beer in certain countries, but not in wine. However, nisin has been reported to act synergistically with sulphites against wine LAB (Rojo-Bezares et al. 2007). Nisin addition could aid to reduce the sulphite content in musts before fermentation. One limitation of nisin addition in certain wines would be inhibition of bacteria responsible for the malolactic fermentation. To solve this shortcoming, nisin-resistant strains of *Oenococcus oeni* have been developed that can grow and maintain malolactic wine fermentation in the presence of nisin.

Pediocins may also find applications in wine. Pediocin N5p from *Pediococcus pentosaceus* is resistant to the physico–chemical factors involved in vinification i.e. pH, temperature, ethanol and SO (Strasser de Saad et al. 1995). Application of pediocin PD-1 produced by *P. pentosaceus* isolated from beer, has been proposed in removal of *O. oeni* biofilms from stainless steel surfaces and also to control growth of *O. oeni* in wine (Bauer et al. 2003). PD-1 was the most effective bacteriocin in removal of an established biofilm from stainless steel surfaces in Chardonnay must when compared with nisin and plantaricin 423 (Nel et al. 2002). *Pediococcus acidilactici* J347-29 produced pediocin PA-1 in presence of ethanol and grape must, suggesting its potential biopreservative in winemaking (Díez et al. 2012). The authors tested the effect of pediocin PA-1 alone and in combination with sulphur dioxide and ethanol on the growth of a collection of 53 oenological LAB, 18 acetic acid bacteria and 16 yeast strains. Acetic acid bacteria and yeasts were not inhibited by pediocin PA-1. *O. oeni* was the most sensitive bacterium compared with other wine

LAB (IC50 values of 19 and 312 ng/ml, respectively). Pediocin inhibitory effect was slightly enhanced in the presence of low concentrations of ethanol (6 %). *O. oeni* was also the most sensitive wine LAB when challenged with lacticin 3147 (García-Ruiz et al. 2013). Inhibitory activity of the bacteriocin was strain-dependent, and one *L. casei* strain was remarkably resistant. A synergistic effect of lacticin 3147 and metabisulphite was demonstrated.

Addition of bacteriocins could prevent spoilage of other alcoholic beverages, such as fermented apple ciders (Galvez et al. 2008; Abriouel et al. 2010). Bacteria causing spoilage of apple juice and fermented apple ciders such as exopolysaccharide-producing lactobacilli (*L. collinoides*, *L. diolivorans* and *P. parvulus* strains) and 3-hydroxypropionaldehyde (3-HPA)-producing *L. collinoides* strains were inhibited efficiently in apple juice and in apple ciders by bacteriocin concentrations in the range of 0.5–5 μg/ml (Grande et al. 2006a; Martínez-Viedma et al. 2008a).

Vegetative cells of the rope-forming strain *B. licheniformis* LMG 19409 (a spoilage strain isolated from spoiled Normand ciders) were rapidly inactivated by bacteriocin addition in fresh-made apple juice and in commercial apple ciders. Although bacterial endospores from *B. licheniformis* were resistant to the bacteriocin, the presence of AS-48 increased the heat sensitivity of endospores. The combination of bacteriocin and moderate heat treatments (85–100 °C) increased the heat inactivation of endospores in cider, decreasing *D* and *z* values (Grande et al. 2006a).

8.2 Bacteriocin-Producing Strains

Inoculation with live cultures is another proposed alternative for inhibition of pathogenic bacteria on fresh produce surfaces (Table 8.1). Bacterial strains (including species of genera such as *Bacillus*, *Pseudomonas*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Weissella*, or *Lactobacillus*) isolated from raw vegetables may produce antagonistic substances to foodborne pathogens (Galvez et al. 2008; Trias et al. 2008a,b). These strains may be better adapted to vegetable substrates and growth under cold or moderate temperatures. The efficacy of such treatments greatly depends on ecological factors such as the capacity to grow and produce antimicrobials in situ by the protective cultures in competition with resident microbiota.

LactiGuard™ (Guardian Food Technologies, LLC) is defined as “bacterial and bacteriological preparations applied to meat, poultry, pork and produce, namely, fresh fruits and vegetables, to decrease pathogens and improve human food safety”. When applied by spray on spinach in combination with water and chlorine as additional hurdles followed by MAP storage 9 days at 4–7 °C, the treatment inhibited *E. coli* O157:H7 and *C. sporogenes* compared to controls with reductions of 1.43 and 1.10 log, respectively (Brown et al. 2011). In freshly harvested spinach, application of the commercial LAB food antimicrobial at 8.0 log CFU/g produced significant ($p < 0.05$) reductions in *E. coli* O157:H7 and *Salmonella* populations on spinach of 1.6 and 1.9 log CFU/g, respectively in the course of aerobic incubation for 12 days at 7 °C (Cálix-Lara et al. 2014).

Fermented vegetables are good sources for isolation of LAB producing bacteriocins. Some examples of antagonistic LAB include *L. lactis* 23 from fermented carrots (Uhlman et al. 1992), *L. plantarum* strains C-11 and C19 from cucumber fermentations (Daeschel et al. 1990; Atrih et al. 1993), *Lactobacillus sake* C2 from traditional Chinese fermented cabbage (Gao et al. 2010), *P. pentosaceus* 05-10 isolated from Sichuan Pickle, a traditionally fermented vegetable product from China (Huang et al. 2009), *L. plantarum* LPCO10 from fermented table olives, (Jimenez-Diaz et al. 1993), *L. plantarum* strains ST23LD and ST341LD from spoiled olive brine (Todorov and Dicks 2005), *Lactobacillus pentosus* B96 from fermenting green olives (Delgado et al. 2005), and *E. faecium* BFE 900 from fermented black olives (Franz et al. 1996). These strains offer potential for investigation as starter or protective cultures in vegetable fermentations, but there are still only limited numbers of studies on this issue.

During cabbage fermentation, inoculation with a paired culture consisting of a nisin-producer *L. lactis* and a nisin-resistant *Ln. mesenteroides* was tested with the purpose of improving the fermentation (Harris et al. 1992a,b). In kimchi, inoculation with a pediocin-producing strain of *P. acidilactici* was reported to successfully achieve inhibition of *L. monocytogenes*, thus improving the product safety (Choi and Beuchat 1994). In a more recent study, kimchi was prepared with *Leuconostoc citreum* GJ7 (producer of the bacteriocin kimchicin GJ7), with the objective of preventing growth and/or survival of foodborne pathogens *E. coli* O157:H7, *Salmonella* Typhi, and *S. aureus* (Chang and Chang 2011). Viable cell reductions of 3.85, 4.45, and 5.19 log CFU/ml were observed 48 h after inoculation. The study concluded that addition of a starter culture capable of producing bacteriocins could serve as a strategy to protect the fermented product from delivering pathogens upon consumption and that the kimchi filtrate itself may be used as a food preservative.

Table olive fermentations are very popular in countries from the Meditarranean region. LAB (mainly *L. plantarum* and *L. pentosus*) together with yeasts are the main bacterial group responsible for these fermentations. These lactobacilli may produce several different bacteriocins (known as plantaricins), and plantaricin genes seem to be widely disseminated (Maldonado et al. 2002). In the Spanish-style process for table olive preparation, green olives are first treated with lye, a treatment that destroys most of the epiphytic microbiota. The lactic fermentation that takes place afterwards is often a slow process that relies mostly on the resident microbiota from the fermentation tanks and manufacturing plant environment. Fast acidification is crucial for proper preservation of olives and inhibition of adventitious microbiota. In some cases, sufficient lactic acid is not produced to warrant product preservation, and spoilage may occur unless exogenous acid is supplemented. The strain *L. plantarum* LPCO10 (plantaricin S and T producer) has been patented for application as a starter culture in the fermentation of table olives and other vegetable foods (Jiménez-Díaz et al. 1993; Ruiz-Barba et al. 1994; Vega Leal-Sánchez et al. 2003). In table olives, inoculation with the plantaricin-producing culture improved the microbiological control of the fermentation process, increased the lactic acid yield and provided a consistent high quality product (Ruiz-Barba et al. 1994; Vega Leal-Sánchez et al. 2003). The starter culture could also be applied to ensure homo-

geneous and faster fermentations in newly-operating plants that still lack the appropriate resident LAB microbiota. *L. plantarum* is also the main bacterial species in other traditional vegetable fermentations such as capers and Almagro eggplants (Seseña et al. 2004; Pérez Pulido et al. 2005). Therefore, plantaricin-producing strains could also find applications in these (and probably others) fermented vegetables.

Cereals and fermented doughs can also be a good source of LAB strains producers of bacteriocins and bacteriocin-like substances, such as bavaricin A, plantaricin ST31, BLIS C57, amylovorin L and others (Messens et al. 2002; Messens and De Vuyst 2002; Narbutaite et al. 2007; Settanni and Corsetti 2008) as well as antifungal compounds (Valerio et al. 2009; Dalié et al. 2010). Although amylovorin is not active against *Bacillus*, amylovorin production may serve to enhance the competitiveness of the producer strain against other lactobacilli in the fermentation (Messens et al. 2002). After a more direct screening for LAB producing bacteriocin-like antirope activities, two strains were selected (*L. plantarum* E5 and *Ln. mesenteroides* A27) that inhibited ropiness in the bread for more than 15 days (Pepe et al. 2003). According to another study, production of antimicrobial activity by sourdough LAB appears to occur at a low frequency, but the producer strains are active in producing antimicrobial activity under sourdough and bread-making conditions (Corsetti et al. 2004). One *L. lactis* strain isolated from raw barley showed a wider inhibitory spectrum than sourdough LAB (Settanni et al. 2005). This strain was found to produce lacticin 3147, and was shown to produce bacteriocin in situ without interfering with growth of bacteriocin-insensitive *Lactobacillus sanfranciscensis* strains. Furthermore, the production of antimicrobial substances, such as reutericyclin and bacteriocins may enhance the competitiveness of strains in fermented doughs (Gänzle and Vogel 2003; Leroy et al. 2007), being considered a desirable trait for starter culture development (Corsetti and Settanni 2007; De Vuyst et al. 2009). It has been suggested that sourdoughs or cultured broths fermented with bacteriocin-producing lactobacilli could be applied to inhibit rope formation by bacilli in yeast-leavened breads (Menteş et al. 2007; Settanni and Corsetti 2008; Valerio et al. 2008).

Ethnic fermented vegetable foods are good candidates for isolation of antagonistic strains producers of (maybe) new bacteriocins, specifically adapted to the particular fermentation conditions of these foods (Kostinek et al. 2007; Yoon et al. 2008; Ge et al. 2009; Hata et al. 2009; Huang et al. 2009; Tamang et al. 2009; Gao et al. 2010). In one study, the nisin-producer *L. lactis* subsp. *lactis* IFO12007 isolated from miso was used as starter for fermentation of cooked rice and rice koji supplemented with soybean extract (Kato et al. 2001). The producer strain proliferated in the cooked rice and produced enough nisin activity to inhibit *B. subtilis* without causing any adverse effect on growth of *Aspergillus oryzae* during the koji fermentation. Furthermore, a lower salt content could be added to rice miso without compromising the lactic acid fermentation of both rice and soybeans (Kato et al. 2001).

Fermented millet flours are widely consumed in many African countries. Yet, the number of studies carried out on their LAB microbiota and their bacteriocins are limited (Ben Omar et al. 2006, 2008). However, results indicate that LAB strains from these fermented foods may have strong inhibitory activities against foodborne pathogens. In one study, the plantaricin-producing strain *L. plantarum* 2.9 (isolated

from ben saalga, a traditional pearl millet fermented food from Burkina Faso) produced strong inhibitory activity in malted millet flour, decreasing the survival of *B. cereus*, *E. coli* O157:H7 and *S. enterica* (Sánchez Valenzuela et al. 2008). The authors of this study suggested that this strain could be applied as a starter culture to improve the microbiological safety of cereal gruels.

Bacteriocin-producing strains can be isolated from raw as well as malted barley. Fermented worts containing bacteriocins could be used to prevent beer spoilage LAB (Vaughan et al. 2005). Bacteriocin production may be a desirable trait for wort bioacidifying LAB starter cultures, enhancing the implantation and proliferation of such strains over spoiling LAB. Development of yeast starter cultures genetically modified to produce bacteriocins has also been suggested, and heterologous production of bacteriocins such as pediocins, leucocins, plantaricins and enterocins by yeasts has been reported (Schoeman et al. 1999; Du Toit and Pretorius 2000; Van Reenen et al. 2002; Gutiérrez et al. 2005; Sánchez et al. 2008; Basanta et al. 2009). The bactericidal yeast strains could be used as starters or protective cultures in the fermentations of brewing, wine and baking processes as biological control agents to inhibit growth of spoilage bacteria.

Several bacteriocinogenic LAB strains have been isolated from wine and wineyards, including species of *L. plantarum*, *O. oeni* and *P. pentosaceus* (Lonvaud-Funel and Joyeux 1993; Strasser de Saad and Manca de Nadra 1993; Navarro et al. 2000; Rojo-Bezares et al. 2007; Knoll et al. 2008; Yanagida et al. 2008). Selected bacteriocin-producing strains could be useful against undesired LAB (such as histamine producers or spoiler) in vinification, and also for proper control of the wine malolactic fermentation (Yurdugül and Bozoglu 2002).

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Chapter 9
Regulations

9.1 The Challenge of Taking Bioprotection Strategies from the Lab to the Market

Application of bioprotection strategies in food preservation may be restricted by laws from different countries that may differ considerably in their fundamentals end practical effects. Some of them are related directly to addition of bioprotectants to foods, but others not less important may be related to apparently secondary aspects such as labelling, packaging, export, use of biological agents or genetically modified organisms.

Licensing antimicrobial preparations as food preservatives is perhaps the most complicated way to follow. Nisin is the only bacteriocin currently approved as a food preservative (E234). Nisin was assessed to be safe for food use by the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives in 1969, and was added to the European food additive list in 1983 (Directive 83/463/EEC; Directive 95/2/EC); (European Economic Community 1983; European Parliament and Council 1995). It was approved in 1988 by the US Food and Drug Agency (FDA) for use in pasteurized processed cheese spreads. The initial approval was followed by other licensed applications (e.g., FSIS 2002). Nisin is legally used in over 80 countries (Adams 2003). However, there are major differences in national legislations concerning the presence and levels of nisin in food products.

Nisin (in the commercial forms Nisaplin™ and Chrisin™) is a lyophilized product obtained from a microbial fermentation. The industrially fermented products are regulated under general food laws. Concentrates or lyophilised powders obtained from fermentates may be added to foods as ingredients. Many commercial preparations currently on the market are sold as ingredients, or shelf-life extenders. Alta™ 2351 and Fargo 23 are natural food ingredients with antilisterial activity produced by bacteriocinogenic strains through a fermentation process. Alta™ 2351 is labelled as "cultured dairy solids (Skim Milk, Dextrose, Whey, and Lactic Acid Culture)."

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It is "an ingredient with functionality against outgrowth of *Listeria* in dairy based products, and small spectrum lactic acid bacteria inhibition." Such products are approved in the US and commercialized in several other countries as well as shelf life extenders for application in a variety of food products.

According to Directive 2000/13/EC (European Parliament and Council 2000) on food labelling, "'ingredient' shall mean any substance, including additives, used in the manufacture or preparation of a foodstuff and still present in the finished product, even if in altered form." Fermented milk or whey concentrates or lyophilised preparations (regardless of whether they contain or not bacteriocins) can be added as ingredients in the preparation of dairy foods. In the European Union, approval of bacteriocins for application as food additives or preservatives must comply with specifications given in Regulation 1333/2008/EC (European Parliament and Council (2008b), which harmonises the use of food additives in foods in the European Community and updates Directive 89/107/EEC (European Commission 1988) concerning food additives authorized for use in foodstuffs intended for human consumption and Directive 95/2/EC (European Parliament and Council 1995) on food additives other than colours and sweeteners, and Regulation 258/97/EC (European Parliament and Council 1997) on novel foods and novel ingredients. Food additives are substances that are not normally consumed as food itself but are added to food intentionally for a technological purpose described in the above Regulation, such as the preservation of food. Preservatives are considered a functional class of food additives: 'preservatives' are substances which prolong the shelf-life of foods by protecting them against deterioration caused by micro-organisms and/or which protect against growth of pathogenic micro-organisms. Approved food additives must be listed in the Community lists and shall specify: (a) the name of the food additive and its E number; (b) the foods to which the food additive may be added; (c) the conditions under which the food additive may be used; (d) if appropriate, whether there are any restrictions on the sale of the food additive directly to the final consumer. Approved specifications should include information to adequately identify the food additive, including origin, and to describe the acceptable criteria of purity. Added preservatives must be listed in food labels with their specific name or EC number (Directive 2000/13/EC; European Parliament and Council 2000). The use and maximum levels of a food additive should take into account the intake of the food additive from other sources and the exposure to the food additive by special groups of consumers (e.g. allergic consumers). The risk assessment and approval of food additives should be carried out in accordance with the procedure laid down in Regulation (EC) No 1331/2008 (European Parliament and Council 2008a) establishing a common authorisation procedure for food additives, food enzymes and food flavourings. Food additives which were permitted before 20 January 2009 shall be subject to a new risk assessment carried out by the Authority.

Bacteriocin preparations could also be applied as processing aids in food manufacture. Directive 2000/13/EC (European Parliament and Council 2000) and Regulation 1333/2008/EC (European Parliament and Council 2008b) do not cover processing aids, but according to Regulation 1333/2008/EC 'processing aid' shall mean any substance which: (1) is not consumed as a food by itself; (2) is intentionally

used in the processing of raw materials, foods or their ingredients, to fulfill a certain technological purpose during treatment or processing; and (3) may result in the unintentional but technically unavoidable presence in the final product of residues of the substance or its derivatives provided they do not present any health risk and do not have any technological effect on the final product. According to this definition, bacteriocins could be applied as processing aids for the preservation of food ingredients, whereby the bacteriocin has no preservative or technological effect in the final food product.

Application of bacteriocins in activated packagings must follow specifications of Directive 2002/72/EC (European Parliament and Council 2002b) concerning plastic materials and articles intended to come into contact with foodstuffs and Regulation (EC) No 1935/2004 (European Parliament and Council 2004) on materials and articles intended to come into contact with food: Active food contact materials are designed to deliberately incorporate 'active' components intended to be released into the food or to absorb substances from the food: "'active food contact materials and articles' (hereinafter referred to as active materials and articles) means materials and articles that are intended to extend the shelf-life or to maintain or improve the condition of packaged food. They are designed to deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food." Substances deliberately incorporated into active materials and articles to be released into the food or the environment surrounding the food shall be authorised and used in accordance with the relevant Community provisions applicable to food, and shall comply with the provisions of this Regulation and its implementing measures. These substances shall be considered as ingredients. Covering or coating materials forming part of the food and possibly being consumed with it (such as edible coatings) do not fall within the scope of this Regulation.

Bacteriocin-producing strains may be applied as starter or bioprotective cultures with the aim of contributing to microbiological safety (Aymerich et al. 2008). For example, Bactoferm F-Lc (Christian Hansen, Denmark) is an antilisterial mixed culture of *Pediococcus acidilactici* and *Lactobacillus curvatus* producing pediocin and sakacin A, respectively for application in fermented sausages. The same company also sells bioprotective cultures containing *Lactobacillus sakei*, and *Leuconostoc carnosum* 4010 for meat products packed under vacuum or modified atmosphere packaging (MAP), and a nisin-producing *Lactococcus lactis* preparation. Danisco (Copenhaguen, Denmark) markets a series of protective cultures (HOLDBAC™) for specific applications in meat and dairy foods based on their capacity to produce bcteriocins as well as other antimicrobial compounds and their competition in food systems. Such preparations include mainly strains of *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *L. sakei*, *Lactobacillus paracasei* and *Propionibacterium freundenreichii* subsp. *shermanii*), whose primary functionalities are growth control of Gram-positive pathogens such as *Listeria*, spoilage microorganisms such as yeasts and moulds, heterofermentative lactic bacteria, and enterococci. Such strains have not been subjected to genetic modification, but the company advertises that local regulations should always be consulted concerning

the status of these products as legislation regarding their use in food may vary from country to country. Similar commercial preparations consisting of bioactive LAb cultures are also available in the US, like for example LactiGuard™, under approval of the US-FDA and the USDA-FSIS.

From a regulatory point of view, bacteriocin-producing strains fall in the category of microbial cultures. In the United States, a new strain of micro-organism for use in food can either be classified as an additive or as a Generally Recognised as Safe (GRAS) substance (Wessels et al. 2004). Food additives are defined in a broad sense as "anything that might come into contact with food (excluding GRAS substances)," and require pre-market approval by the US FDA based on toxicological and efficacy data. The consideration of GRAS status is based on the availability of enough information relevant to the substance safe use for a given intended purpose: "generally recognized, among experts qualified by scientific training and experience to evaluate its safety, as having been adequately shown through scientific procedures (or, in the case as a substance used in food prior to January 1, 1958, through either scientific procedures or experience based on common use in food) to be safe under the conditions of its intended use" (US Food and Drug Administration 1999). The intended use is an essential part of the GRAS status concept. For instance, the GRAS status of a given strain for use in a yogurt product is not valid for the same strain in infant formulae (Wessels et al. 2004). The GRAS status is determined by qualified experts, not by the FDA. The food company that uses the bacterium assumes complete responsibility, regardless of its GRAS status.

Within the European Union, microbial food cultures with a long history of safe use are considered as traditional food ingredients, and covered by general European food law (Regulation 178/2002/EC; European Parliament and Council 2002a). Microbial cultures must also be safe for their intended use. Novel use of microbial cultures is regulated in Regulation 258/97/EC (European Parliament and Council 1997) if a microorganism has not been consumed in a significant degree before May 15, 1997. This may apply to selected bacteriocin producer strains isolated from a source different than the food where they will be applied. There is also an ongoing dispute in Europe regarding the food category of starter cultures with protective properties, since they may be considered as cultures with specific technological effects (preservatives). This may contradict current regulations on approval of new preservatives, given the long history of consumption of fermented foods and the fact that the original and primary purpose of fermenting food was to achieve a preservation effect. In this respect, guidance documents from the European Food Safety Authority (EFSA 2007, 2008) established a pre-market safety assessment of selected groups of microorganisms leading to a "Qualified Presumption of Safety (QPS)" if the taxonomic group did not raise safety concerns or, if safety concerns existed, but could be defined and excluded (the qualification) the grouping could be granted QPS status. Thereafter, any strain of microorganism the identity of which could be unambiguously established and assigned to a QPS group would be freed from the need for further safety assessment other than satisfying any qualifications specified. Microorganisms not considered suitable for QPS would remain subject to a full safety assessment.

Application of genetically-modified bacteriocin producer strains on foods may be affected by different EU regulations and Directives. Strains modified by naturally-existing procedures (such as DNA transformation or plasmid conjugation) could be applied in foods with no other restrstions than those specified in previous paragraph for naturally-occurring strains. However, application of strains modified by procedures involving extensive DNA manipulation and artificial transfer to recipient cells is under much more strict control, including the specifications and limitations established by Directive 2001/118/EC (European Parliament and Council 2001) on the deliberate release of GMMs into the environment, Directive 2009/41/EC (European Parliament and Council 2009) on the contained use of genetically modified micro-organisms, and Regulation 1829/2003/EC (European Parliament and Council 2003) concerning the marketing of GMOs intended for food or feed and of food or feed products containing, consisting of, or produced from GMOs. Bacterial products such as fermented bio-active ingredients prepared from GMM should also need approval in accordance with specifications under Regulations1829/2003/EC and 1333/2008/EC (European Parliament and Council 2003, 2008a, b).

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