Bacteriocin-based strategies for food biopreservation

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Abstract

Bacteriocins are ribosomally-synthesized peptides or proteins with antimicrobial activity, produced by different groups of bacteria. Many lactic acid bacteria (LAB) produce bacteriocins with rather broad spectra of inhibition. Several LAB bacteriocins offer potential applications in food preservation, and the use of bacteriocins in the food industry can help to reduce the addition of chemical preservatives as well as the intensity of heat treatments, resulting in foods which are more naturally preserved and richer in organoleptic and nutritional properties. This can be an alternative to satisfy the increasing consumers demands for safe, fresh-tasting, ready-to-eat, minimally-processed foods and also to develop “novel” food products (e.g. less acidic, or with a lower salt content). In addition to the available commercial preparations of nisin and pediocin PA-1/AcH, other bacteriocins (like for example lacticin 3147, enterocin AS-48 or variacin) also offer promising perspectives. Broad-spectrum bacteriocins present potential wider uses, while narrow-spectrum bacteriocins can be used more specifically to selectively inhibit certain high-risk bacteria in foods like Listeria monocytogenes without affecting harmless microbiota. Bacteriocins can be added to foods in the form of concentrated preparations as food preservatives, shelf-life extenders, additives or ingredients, or they can be produced in situ by bacteriocinogenic starters, adjunct or protective cultures. Immobilized bacteriocins can also find application for development of bioactive food packaging. In recent years, application of bacteriocins as part of hurdle technology has gained great attention. Several bacteriocins show additive or synergistic effects when used in combination with other antimicrobial agents, including chemical preservatives, natural phenolic compounds, as well as other antimicrobial proteins. This, as well as the combined use of different bacteriocins may also be an attractive approach to avoid development of resistant strains. The combination of bacteriocins and physical treatments like high pressure processing or pulsed electric fields also offer good opportunities for more effective preservation of foods, providing an additional barrier to more refractile forms like bacterial endospores as well. The effectiveness of bacteriocins is often dictated by environmental factors like pH, temperature, food composition and structure, as well as the food microbiota. Foods must be considered as complex ecosystems in which microbial interactions may have a great influence on the microbial balance and proliferation of beneficial or harmful bacteria. Recent developments in molecular microbial ecology can help to better understand the global effects of bacteriocins in food ecosystems, and the study of bacterial genomes may reveal new sources of bacteriocins.

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1. Introduction

In spite of modern advances in technology, the preservation of foods is still a debated issue, not only for developing countries (where implementation of food preservation technologies are clearly needed) but also for the industrialized world. Amelioration of economic losses due to food spoilage, lowering the food processing costs and avoiding transmission of microbial pathogens through the food chain while satisfying the growing consumers demands for foods that are ready to eat, fresh-tasting, nutrient and vitamin rich, and minimally-processed and preserved are major challenges for the current food industry. The extent of microbiological problems in food safety was clearly reflected in the WHO food strategic planning meeting (WHO, 2002): (i) the emergence of new pathogens and pathogens not previously associated with food consumption is a major concern; (ii) microorganisms have the ability to adapt and change, and changing modes of food production, preservation and packaging have therefore resulted in altered food safety hazards.

The empirical use of microorganisms and/or their natural products for the preservation of foods (biopreservation) has been a common practice in the history of mankind (Ross et al., 2002). The lactic acid bacteria (LAB) produce an array of antimicrobial
substances (such as organic acids, diacetyl, acetoin, hydrogen peroxide, reuterin, reutericyclin, antifungal peptides, and bacteriocins (Holzapfel et al., 1995; El-Ziney et al., 2000; Holtzel et al., 2000; Magnusson and Schnürer, 2001). Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins (Jack et al., 1995). LAB produce a variety of bacteriocins, most of which can be grouped in one of the classes proposed by Klaenhammer (1993). The structure, biosynthesis, genetics and food application of LAB bacteriocins have been reviewed recently (Cleveland et al., 2001; O’Sullivan et al., 2002; Chen and Hoover, 2003; Cotter et al., 2005; Finland et al., 2005; Deegan et al., 2006; Drider et al., 2006).

The bacteriocins produced by LAB offer several desirable properties that make them suitable for food preservation: (i) are generally recognised as safe substances, (ii) are not active and non-toxic on eukaryotic cells, (iii) become inactivated by digestive proteases, having little influence on the gut microbiota, (iv) are usually pH and heat-tolerant, (v) they have a relatively broad antimicrobial spectrum, against many food-borne pathogenic and spoilage bacteria, (vi) they show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane: no cross resistance with antibiotics, and (vii) their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation.

The accumulation of studies carried out in recent years clearly indicate that the application of bacteriocins in food preservation can offer several benefits (Thomas et al., 2000): (i), an extended shelf life of foods, (ii) provide extra protection during temperature abuse conditions, (iii) decrease the risk for transmission of foodborne pathogens through the food chain, (iv) ameliorate the economic losses due to food spoilage, (v) reduce the application of chemical preservatives, (vi) permit the application of less severe heat treatments without compromising food safety: better preservation of food nutrients and vitamins, as well as organoleptic properties of foods, (vii), permit the marketing of “novel” foods (less acidic, with a lower salt content, and with a higher water content), and (viii) they may serve to satisfy industrial and consumers demands. In this respect some of the trends of the food industry in Europe, such as the need to eliminate the use of artificial ingredients and additives, the demands for minimally-processed and fresher foods, as well as for ready-to-eat food or the request for functional foods and nutraceuticals (Robertson et al., 2004) could be satisfied, at least in part, by application of bacteriocins. The present review will address different aspects related to food preservation by bacteriocins including factors influencing bacteriocin activity in food systems, hurdle technology, and the impact of recent advances in molecular biology and the analysis of bacterial genomes on bacteriocin studies and application.

2. Bacteriocins and food systems

2.1. Supplemeting foods with bacteriocins

Foods can be supplemented with ex situ produced bacteriocin preparations, or by inoculation with the bacteriocin-producer strain under conditions that favour production of the bacteriocin in situ (Schillinger et al., 1996; Stiles, 1996). In the first case, bacteriocin preparations obtained by cultivation of the producer strain in a fermentor at industrial scale followed by adequate recovery and processing can be added as partially purified or purified concentrates, which would require specific approval as preservatives from the legislative point of view. So far, nisin is the only bacteriocin licensed as a food preservative (E234). Many preliminary studies on the activity of bacteriocins in vitro or in food systems are carried out with partially-purified preparations obtained from cultured broths. In most cases, a low concentration of bacteriocin is often recovered, which limits the efficacy of such preliminary tests.

Ex situ produced bacteriocins can also be added in the form of raw concentrates obtained by cultivation of the producer strain in a food-grade substrate (such as milk or whey). The resulting preparations may be regarded as food additives or ingredients from the legal point of view, since some of their components may play a recognised function in the food (such as increase in protein content, or thickening). They also contain the cell-derived antimicrobial metabolites (such as lactic acid) and bacteriocins, affording an additional bioprotectant function. In addition to already-marketed concentrates such as ALTA™ 2341 or Microgard™, other milk-based preparations have been described recently such as lacticin 3147 (Morgan et al., 1999; Guinane et al., 2005) and variacin (O’Mahony et al., 2001).

Ex situ produced bacteriocins can also be applied in the form of immobilized preparations, in which the partially-purified bacteriocin or the concentrated cultured broth is bound to a carrier. The carrier acts as a reservoir and diffuser of the concentrated bacteriocin molecules to the food ensuring a gradient-dependent continuous supply of bacteriocin. The carrier may also protect the bacteriocin from inactivation by interaction with food components and enzymatic inactivation. Moreover, the precise localized application of bacteriocin molecules on the food surface requires much lower amounts of bacteriocin (compared to application in the whole food volume), decreasing the processing costs. A variety of methods have been proposed for bacteriocin immobilization, including adsorption to the producer cells (Yang et al., 1992; Mattila et al., 2003; Ghalfi et al., 2006), silica particles or corn starch powder (Coventry et al., 1996; Dawson et al., 2005), liposome encapsulation (Degnan and Luchansky, 1992), and incorporation on gel coatings and films of different materials such as calcium alginate, gelatin, cellulose, soy protein, corn zein, collagen casings, polysaccharide based films, cellophane, silicone coatings, polyethylene, nylon or other polymer plastic films (Daeschel et al., 1992; Cutter and Siragusa, 1995b; Ming et al., 1997; Siragusa et al., 1999, Natraj and Sheldon, 2000; Gill and Holley, 2003; Scannell et al., 2000a; Ko et al., 2001; Dawson et al., 2002; Franklin et al., 2004; Luchansky and Call, 2004; Guerra et al., 2005; Lungu and Johnson, 2005). In most cases, immobilized bacteriocin preparations are applied on the surface of the processed food to avoid post-process contamination and surface proliferation of unwanted bacteria. A recent advance in this field is the use of immobilized bacteriocins in the development of antimicrobial packaging. A polyethylene film containing immobilized bacteriocin 32Y from L. curvatus reduced viable counts of L. monocytogenes during storage in the packaged pork steak and ground beef as well as in frankfurters (Mauriello et al.,...
Similarly, a nisin-containing cel-lophane coating reduced viable counts of total aerobic bacteria in fresh veal meat stored at 8 °C (Guerra et al., 2005), and an active package obtained from nisin-treated film reduced viable counts of *M. luteus* ATCC 10,240 cells in broth as well as in raw milk and pasteurized milk during storage (Mauriello et al., 2005). Therefore, the use of antimicrobial films containing bacteriocins can improve the quality and safety and prolong the shelf-life of food products.

In situ bacteriocin production offers several advantages compared to ex situ production regarding both legal aspects and costs. Lowering the costs of biopreservation processes may be highly attractive, especially for small economies and developing countries, where food safety may be seriously compromised (Holzapfel, 2002). Many studies have focused on the selection and development of bacteriocinogenic cultures for food application (Ross et al., 2000; Työppönen et al., 2003; Peláez and Requena, 2005; Foulquié Moreno et al., 2006; Leroy et al., 2006). The use of bacteriocinogenic cultures requires careful selection of strains that are well-adapted to the particular food environment in which they will be used and able to grow under the food processing and/or storage conditions and to produce enough bacteriocin amounts as to inhibit the target pathogenic or spoilage bacteria. Therefore, it is necessary to implement the right experimental approaches to select bacteriocin-producing strains that are suitable for use in food production. The strain properties and the amount of bacteriocin produced could be improved by heterologous expression of bacteriocin genes (Rodriguez et al., 2003; Zhou et al., 2006), and the precise moment of bacteriocin production could also be tailored by using inducible production systems (Zhou et al., 2006).

Bacteriocinogenic strains can be used either directly as starter cultures, as adjunct or co-cultures in combination with a starter culture, or as protective cultures (especially in the case of non-fermented foods). When used as a starter culture, the bacteriocinogenic strain must be able to carry out the desired fermentation process optimally besides being able to produce enough bacteriocin amounts to afford protection. In some cases, bacteriocin production may also serve to increase the implantation capacity, competitiveness and stability of the starter (Todorov et al., 1999). Adjunct cultures do not need to contribute to the fermentation, but they must not interfere with the primary function of the starter culture. For this reason, bacteriocin resistance of the starter culture may be a key factor. This may be achieved by selection of natural resistant mutants, by adaptation through repeated subcultivation with increasing bacteriocin concentrations, or by genetic modification. Nevertheless, sometimes this may not be necessary as the bacteriocin may just not be active on the starter culture (as may be the case of many of the bacteriocins that predominantly show antilisterial activity) or this may be much more tolerant to the bacteriocin than the target bacteria in the food system. Differences in inoculum density, a faster growth rate of the starter or a delayed bacteriocin production may also permit the starter to grow without interference from the bacteriocinogenic adjunct culture. As an example, inoculation of milk with an enterocin AS-48 producer enterococcal strain as adjunct culture in combination with a commercial starter culture for cheese manufacture had no effect on growth of the starter or the physicochemical properties of the produced cheese. At the same time, enough bacteriocin was produced in the cheese to ensure inhibition of *Bacillus cereus* (Muñoz et al., 2004).

Bacteriocinogenic protective cultures can be used to inhibit spoilage and pathogenic bacteria during the shelf life period of non-fermented foods. A protective culture may grow and produce bacteriocin during refrigeration storage of the food, and/or during temperature abuse conditions. In the first case, growth of the protective cultures must have a neutral impact on the physicochemical and organoleptic properties of the food, while under temperature abuse conditions the protective culture may even act as the predominant spoiler, ensuring that pathogenic bacteria do not grow and that the spoiled food is not consumed (Holzapfel et al., 1995).

2.2. Factors influencing the efficacy of bacteriocins in food systems

Comparisons of data obtained in culture media with those obtained in food systems reveal that the efficacy of bacteriocins is often much lower in the later (Schillinger et al., 1996). Sometime, at least ten-fold higher bacteriocin concentrations must be added to foods in order to achieve an equivalent inhibitory effect. The efficacy of bacteriocins in foods will greatly depend on a number of food-related factors (Table 1) that in most cases involve interaction with food components, precipitation, inactivation, or uneven distribution of bacteriocin molecules in the food matrix. As an illustrative example, application of nisin in meat products faces several limitation derived from its interaction with phospholipids emulsifiers and other food constituents (Henning et al., 1986; Jung et al., 1992; Aasen et al., 2003), poor solubility at pH above 6.0, and inactivation by formation of a nisin-glutathione adduct (Rose et al., 2003). However, inactivation is

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lower in cooked meats due to the loss of free sulphhydryl groups during cooking as a result of the reaction of glutathione with proteins (Stergiou et al., 2006).

Foods are complex ecosystems with a range of microbial compositions. These vary from (commercially) sterile foods to raw or fermented foods. In commercially sterile foods, microorganisms from post-process contamination may easily proliferate because of the lack of competitors. Under these conditions, the efficacy of the bacteriocin will depend more directly on the microbial load of the contaminant, and a higher bacteriocin concentration will always be required in order to inactivate a higher number of target cells. In raw foods, the autochthonous microbiota may counteract development of potential pathogens. However, a complex food microbiota may frequently cause a lower efficacy of bacteriocins due to the presence of resistant bacteria (such as Gram-negatives) and/or the higher chances for production of inactivating enzymes (such as proteases). In addition, the bacteriocin may not always target the desired bacterial group (for example, bacteriocin inactivation of the LAB responsible for food fermentation would be clearly undesirable). In processed foods, microorganisms can also be found in a variety of physiological stages, which may greatly influence bacteriocin sensitivity. As an example, cells that are not actively growing may be more resistant to bacteriocins, and changes of the target organisms in response to environmental stress factors may also result in decreased bacteriocin sensitivity. Inactive bacterial forms (endospores) may also be resistant to bacteriocins, although processing treatments may trigger spore germination and outgrowth, increasing bacteriocin sensitivity. Therefore, the efficacy of bacteriocins in food will greatly depend on the food microbial composition and microbial physiological stage (Table 1). It should also be considered that microorganisms are seldom distributed homogeneously as planktonic cells in the food matrix. Most probably, they will tend to form microcolonies in a solid or particulate food, or grow in the form of slime-covered biofilms on food surfaces. The protective effect of biofilms against antimicrobial substances in the food industry is well documented (Kumar and Anand, 1998), and may clearly limit the efficacy of bacteriocins as well.

Variations in strain sensitivities and development of strain resistance/adaptation are also of major concern for application of bacteriocins. For example, not all strains of L. monocytogenes show the same degree of sensitivity to antilisterial bacteriocins (Martínez et al., 2005). Bacteriocin-resistant L. monocytogenes have been reported to appear at frequencies of 10$^{-3}$ to 10$^{-9}$ for nisin, depending on the strain (Ming and Daeschel, 1993; Davies and Adams, 1994; Mazzotta and Montville, 1997; Martínez et al., 2005), of 10$^{-4}$ to 10$^{-6}$ for the class IIa bacteriocins leucocins A, B, E and sakacin A (Dykes and Hastings, 1998), and of 10$^{-3}$ to 10$^{-4}$ for mesenterocin 52, curvaticin 13 and plantaricin C19 (Rekhif et al., 1994). The fitness costs of bacteriocin resistance may vary greatly depending on the strain, the bacteriocin and the environmental conditions (Gravesen et al., 2002a). Most worrying, the developed resistance to one bacteriocin may also afford protection against other bacteriocins, and the observed cross-resistance between pediocin-like bacteriocins has been attributed to a general mechanism of resistance (Gravesen et al., 2002b). The needs to find novel bacteriocins showing no cross resistance with existing ones are clearly patent.

In addition to the factors influencing the effectiveness of bacteriocins as antimicrobials in food systems, factors influencing bacteriocin production are of most importance when using bacteriocinogenic cultures (Fig. 1). The use of bacteriocinogenic cultures faces several limitations directly related to the producer strain such as (i) the spontaneous loss of the bacteriocinogenic trait, (ii) the susceptibility of the producer strain to infection by bacteriophages, (iii) antagonism of other bacteria towards the producer strain, (iv) inadequacy of producer strain as a starter, (v) difficulties for genetic manipulation and transfer of bacteriocinogenic trait to suitable starters, and, the most important, (vi) a low capacity for bacteriocin production in the food system (which will depend greatly on the food storage conditions, the capacity of the producer strain for implantation and proliferation, or the induction of bacteriocin production). In situ bacteriocin production may also depend greatly on physicochemical factors (such as pH, temperature, aw, CO$_2$, O$_2$, redox potential, time of incubation...) as well as food-related factors (such as the food structure — fluidity, particulate matter, emulsions..., —
buffering capacity, composition — available nutrients, additives, antimicrobials... — and processing conditions — freezing and thawing, pressure homogenization or other procedures that may indirectly damage bacterial cells, as well as thermal treatments and other treatments intended to reduce the microbial load. Since the bacteriocinogenic strain will thrive within a (more or less complex) microbial population in the food ecosystem, bacteriocin production may also depend greatly on microbial-related factors such as the microbial load and diversity, the microbial interactions occurring in the food during storage (such as the competition for nutrients or the production of other antagonistic substances), and the physiological state of the bacteria (Holzapfel et al., 1995; Rodriguez et al., 2003; Devlieghere et al., 2004). All these factors will be sensed by the bacteriocinogenic strain which, in turn, will also have a direct influence on the food environment through the consumption of and competition for nutrients, production of metabolites and modification of the food microbial balance through the produced bacteriocin. Therefore, bacteriocin production in food must be understood as a dynamic process where the different interactions change over time, dictating the ultimate result as far as food preservation (Fig. 1).

Several illustrative examples may explain how bacteriocin production can change dramatically by altering environmental conditions, and how optimum production may require a certain combination of influencing factors (Leal-Sánchez et al., 2002). A suboptimal temperature (22 °C) and a moderate NaCl stress (0.65 M) stimulated bacteriocin production by Lactobacillus pentosus B96 (Delgado et al., 2005). Amylovorin production by Lactobacillus amylovorus DCE 471 was stimulated by NaCl (Neyesens et al., 2003) as well as carbon dioxide (Neyesens and De Vuyst, 2005). Several other stress factors (such as ethanol, oxygen, competing microbiota, etc.) may also stimulate bacteriocin production.

Bacteriocin production is often an inducible trait that depends on cell density and concentration of the inducer (which may be the bacteriocin itself). Therefore, a minimum concentration of inoculum is often required. The interactions of the induction factor with the food matrix (such as adsorption or inactivation) may have a great influence on bacteriocin production. On the other hand, the food matrix may also facilitate the concentration of the induction factor around cells or microcolonies formed by the bacteriocinogenic culture in the food. The presence of competing microorganisms can be an environmental factor stimulating production of LAB bacteriocins such as lactacin B (Barefoot et al., 1994) or dervercin (Sip et al., 1998). Similarly, specific Gram-positive bacteria activate both plantaricin NC8 (PLNC8) production by Lactobacillus plantarum NC8 and the PLNC8-autoinducing activity (Maldonado et al., 2004).

Since bacteriocin production is linked to cell growth, it may also depend on factors affecting this parameter (such as inhibitory substances like salt or nitrite) or the lack of available nutrients (such as manganese in the case of many LAB). As an example, the production of enterocins A and B by Enterococcus faecium CTC492 was significantly inhibited by sausage ingredients and additives, with the exception of nitrate (Aymerich et al., 2000). The addition of sodium chloride and pepper decreased production by 16-fold. The temperature and pH influenced enterocin production, with optima between 25 and 35 °C, and from 6.0 to 7.5 of initial pH (Aymerich et al., 2000). Bavacin A production was negatively affected at NaCl concentrations of 3% or higher, while cells were still growing (Larsen et al., 1993). Growth and sakacin production in meat sausages by Lactobacillus sakei CTC 494 was affected negatively by salting and curing with nitrite as well as manganese limitation (Leroy and De Vuyst, 1999, 2005). Similarly, growth and curvacin A production by Lactobacillus curvatus LTH 1174 were negatively affected by nitrite (even by a concentration as low as 10 ppm), and also by sodium chloride (Verluytten et al., 2003, 2004a). The effect on bacteriocin production was even detected at low NaCl concentrations where there was no growth inhibition, and NaCl was shown to interfere with bacteriocin induction (Verluytten et al., 2004a). Growth and curvacin A production were also negatively affected by some species (especially by nutmeg) in a meat system (Verluytten et al., 2004b). A model was proposed to illustrate the influence of environmental factors on sakacin production in a meat system (Leroy and De Vuyst, 2003, 2005).

3. Bacteriocins and hurdle technology

The concept of hurdle technology began to apply in the food industry in a rational way after the observation that survival of microorganisms greatly decreased when they were confronted with multiple antimicrobial factors (Leistner, 1978; Leistner and Gorris, 1995; Leistner, 2000). After exposure of a bacterial population to a single antimicrobial factor there is often a heterogeneous response, depending on the intensity of treatment as well as many other factors. A fraction of the population may receive a lethal dose of the antimicrobial factor, leading to cell death. The remaining fraction may survive due to several reasons: (i) receiving a sub-lethal dose; (ii) showing an increased resistance because of its physiological state (e.g. stationary phase cells, or cells already stressed in response to other unfavourable environmental conditions), and (iii) cells naturally resistant to the antimicrobial agent. Sub-lethally injured cells as well as cells with increased resistance may repair the damage caused by the antimicrobial agent and survive, having a good opportunity to develop mechanisms of resistance or adaptation that would make them immune to further challenges with the particular antimicrobial factor. By contrast, when cells are exposed to a combination of antimicrobial factors, the intensity of damage may be higher since some of the antimicrobial factors may act on the same cellular target. The repair of multiple damages may also require much higher energy costs, leading to energy exhaustion and cell death. Therefore, the probabilities for survival and proliferation for cells confronted with multiple hurdles are very low. In addition, the synergy between different antimicrobial factors may allow the use of lower doses compared to their individual application.

Over 60 potential hurdles have been described to improve food stability and/or quality (Leistner, 1999). The application of bacteriocins as part of hurdle technology has received great attention in recent years (Chen and Hoover, 2003; Ross et al.,...
2003; Deegan et al., 2006), since bacteriocins can be used purposely in combination with selected hurdles in order to increase microbial inactivation (Fig. 2). The combination of hurdles to be applied will depend greatly on the type of food and its microbial composition. This must be carefully considered, since different hurdles usually have different effects on the members of a microbial community. As an example, food acidification may select for aciduric bacteria while heat treatment may select for endospore formers. Also, elimination of some members of the population may provide a more favourable environment for others, due to the lack of competition. The interaction of different antimicrobial factors may also modify their individual antimicrobial spectra. For example, Gram-negative bacteria may become sensitized to bacteriocins and other molecules upon exposure to hurdles that destabilise the bacterial outer membrane, as will be discussed further on.

3.1. Combination of bacteriocins with chemical substances and natural antimicrobials

Previous studies have shown that the presence of NaCl enhanced the antimicrobial action of bacteriocins such as nisin, leucocin F10, enterocin AS-48 and others (Harris et al., 1991; Thomas and Wimpenny, 1996; Mazzotta et al., 1997; Parente et al., 1998; Ananou et al., 2004). However, nisin activity was also antagonized by low concentration of NaCl (Boutefroy et al., 2000). Sodium chloride also decreased the antilisterial activity of acidocin CH5 (at 1–2%; Chumchalová et al., 1998), lactocin 705 (at 5–7%; Vignolo et al., 1998), leucocins 4010 (at 2.5% NaCl; Hornbæk et al., 2004), pediocin (at 6.5% NaCl; Jydegaard et al., 2000), curvacin (Verluyten et al., 2002) and Carnobacterium piscicola A9b bacteriocin (at 2–4% NaCl; Himmelbloom et al., 2001). The protective effect of sodium chloride may be due to interference with ionic interactions between bacteriocin molecules and charged groups involved in bacteriocin binding to target cells (Bhunia et al., 1991). Sodium chloride may also induce conformational changes of bacteriocins (Lee et al., 1993) or changes in the cell envelope of the target organisms (Jydegaard et al., 2000).

Reduction of nitrite content by addition of bacteriocins may be beneficial in the food industry. The combinations of nisin and nitrite delayed botulinal toxin formation in meat systems and showed increased activity on clostridial endospores outgrowth (Rayman et al., 1981, 1983; Taylor et al., 1985) and also on Leuconostoc mesenteroides and L. monocytogenes (Gill and Holley, 2003). Addition of nitrite also increased the anti-listeria activity of bacteriocinogenic lactobacilli in meat (Hugas et al., 1996) and the activities of enterocin EJ97 against L. monocytogenes, Bacillus coagulans and Bacillus macroides (García et al., 2003, 2004a,b) and enterocin AS-48 against B. cereus (Abriouel et al., 2002).

Organic acids and their salts can potentiate the activity of bacteriocins greatly, while acidification enhances the antibacterial activity of both organic acids and bacteriocins (Jack et al., 1995; Stiles, 1996). The increase in net charge of bacteriocins at low pH might facilitate translocation of bacteriocin molecules through the cell wall. The solubility of bacteriocins may also increase at lower pH, facilitating diffusion of bacteriocin molecules. Buncic et al. (1995) found that the sensitivity of L. monocytogenes to nisin (400 IU/ml) increased in combination with lactate. Further reports have confirmed the increased antibacterial activity of nisin in combination with sodium lactate in several food systems (Scannell et al., 1997; Nykänen et al., 2000; Long and Phillips, 2003; Ukuku and Fett, 2004). A nisin-sorbate combination showed increased activity against Listeria (Avery and Buncic, 1997), and B. licheniformis (Mansour et al., 1998). In the production of Ricotta-type cheeses, the combination of nisin with acetic acid and sorbate controlled L. monocytogenes contamination over a long period storage (70 days) at 6–8 °C (Davies et al., 1997). Lacticin 3147 activity also increased in combination with sodium lactate or sodium citrate (Scannell et al., 2000b), as well as did pediocin AcH activity in combination with sodium diacetate and sodium lactate (Uhart et al., 2004). Activity of enterocin AS-48 against B. cereus in

Fig. 2. Application of bacteriocins as part of hurdle technology.
rice gruel was also potentiated by sodium lactate (Grande et al., 2006). Lactic acid, sodium lactate and peracetic acid (as well as several other chemical compounds) increased AS-48 activity for decontamination of L. monocytogenes in sprouts (Cobo Molinos et al., 2005). When nisin and pediocin were used in combination with chemical compounds to combat L. monocytogenes in fresh produce, best results were obtained for nisin with phytic acid (Bari et al., 2005).

Chelating agents permeate the outer membrane (OM) of Gram-negative bacteria by extracting Ca^{2+} and Mg^{2+} cations that stabilize lipopolysaccharide of this structure, allowing bacteriocins to reach the cytoplasmic membrane (Stevens et al., 1991; Vaara, 1992; Schved et al., 1994; Helander et al., 1997). The enhanced effect of chelators such as EDTA, disodium pyrophosphate, trisodium phosphate, hexametaphosphate or citrate and bacteriocins against Gram-negative bacteria has been demonstrated for nisin both under laboratory conditions and in foods (Stevens et al., 1991; Cutter and Siragusa, 1995a,b; Carneiro De Melo et al., 1998; Bozias and Adams, 1999; Fang and Tsai, 2003). Brochracin C and enterocin AS-48 also showed increased antimicrobial activity on EDTA-treated Gram-negative bacteria (Abriouel et al., 1998; Gao et al., 1999; Ananou et al., 2005). Sensitization of Gram-negative bacteria to bacteriocins by other chelators such as maltol or ethyl maltol has been reported (Schved et al., 1996). Sodium lactate or sodium citrate in combination with nisin showed increased antimicrobial activity against Arcobacter butzleri in chicken due to their chelating effect (Long and Phillips, 2003). Chelating agents can also enhance the activity of bacteriocins on Gram-positive bacteria. A combination of nisin and sodium polyphosphate showed an increased activity against L. monocytogenes (Buncic et al., 1995). More recently, an increased activity of chrisin (a commercial nisin preparation) in combination with EDTA has been reported (Schved et al., 1996). Sodium lactate or sodium citrate in combination with nisin showed increased antimicrobial activity against Arcobacter butzleri in chicken due to their chelating effect (Long and Phillips, 2003). Chelating agents can also enhance the activity of bacteriocins on Gram-positive bacteria. A combination of nisin and sodium polyphosphate showed an increased activity against L. monocytogenes (Buncic et al., 1995). More recently, an increased activity of chrisin (a commercial nisin preparation) in combination with EDTA has been reported (Schved et al., 1996).

Sub-lethal concentrations of nisin (30 IU/ml) and monolaurin (100 µg/ml) in combination acted synergistically on B. licheniformis vegetative cells and spore outgrowth in milk (Mansour et al., 1999). Synergism was also observed for the sucrose fatty acid esters sucrose palmitate and sucrose stearate and nisin against several strains of L. monocytogenes, B. cereus (cells and spores), L. plantarum and Staphylococcus aureus, but not against Gram-negative bacteria (Thomas et al., 1998). Reuterin also showed a significant synergistic effect on L. monocytogenes and a slight additive effect on S. aureus after in combination with nisin (100 IU/ml), although the antimicrobial effect of reuterin against Gram-negative pathogens was not enhanced, though (Arquès et al., 2004).

Essential oils and their active components, the phenolic compounds are also attractive natural preservatives (Burt, 2004). When used in combination with bacteriocins, the dose of added phenolic compounds could be lowered thereby decreasing their impact on the food flavour and taste. Nisin acted synergistically with carvacrol, eugenol or thymol against B. cereus and/or L. monocytogenes (Pol and Smid, 1999; Periago et al., 2001; Yamazaki et al., 2004). Combinations of nisin with carvacrol, eugenol, or thymol resulted in synergistic action against Bacillus subtilis and Listeria innocua, while nisin and cinnamon acid had synergistic activity against L. innocua, but only additive against B. subtilis (Olasupo et al., 2004). Carvacrol (0.5 mM) was used to enhance the synergy found between nisin and a pulsed electric field treatment (PEF) against vegetative cells of B. cereus in milk (Pol et al., 2001a,b). The combination of nisin and cinnamon accelerates death of Salmonella Typhimurium and Escherichia coli O157:H7 in apple juice (Yuste and Fung, 2004). The natural variant nisin Z also acted synergistically with thymol against L. monocytogenes and B. subtilis (Ettayebi et al., 2000). The antimicrobial activity of enterocin AS-48 against S. aureus cell in vegetable sauces was potentiated significantly in combination with the phenolic compounds carvacrol, geraniol, eugenol, terpineol, caffeic acid, p-coumaric acid, citral and hydrocinnamic acid (Grand et al., 2007).

Combinations of bacteriocins have also been tested in order to increase their antimicrobial activities. The simultaneous use of nisin with pediocin AcH (Hanal et al., 1993) or with leucocin F10 (Parente et al., 1998) as well as lactacin B or lactacin F with nisin or pediocin AcH, and lactacin 481/pediocin AcH (Mulet-Powell et al., 1998) provides a greater antibacterial activity than each bacteriocin separately. Simultaneous or sequential additions of nisin (50 IU/ml) and curvatinic 13 (160 AU/ml) also induced a greater inhibitory effect against L. monocytogenes than each bacteriocin individually (Boutefroy and Millière, 2000). The simultaneous use of two or more bacteriocins could be useful not only to lower the added bacteriocin doses, but also to avoid regrowth of bacteriocin-resistant/adapted cells. However, development of cross-resistance should be carefully considered, especially for the combinations of bacteriocins belonging to the same class, as it has been shown that a general mechanism may account for high-level resistance to class Ila bacteriocins in L. monocytogenes (Gravesen et al., 2002a,b).

An increased antibacterial activity can also be achieved by combination of bacteriocins with other (non-bacteriocin) antimicrobial proteins or peptides. Nisin and lysozyme acted synergistically against Gram-positive bacteria, including spoilage lactobacilli and S. aureus (Chung and Hancock, 2000; Nattress and Baker, 2003). The spectrum of the nisin-lysozyme combination could be extended to Gram-negative bacteria by addition of chelating agents (Gill and Holley, 2000) The combined addition of nisin and the lactoperoxidase system (LPS) had a strong antimicrobial effect against L. monocytogenes Ohio and (to a lesser extent) L. monocytogenes Scott A in skim milk after 24 h at 30 °C (Zapico et al., 1998), against L. monocytogenes ATCC 15313 in skim milk at 25 °C for at least 15 days (Boussouel et al., 2000), and also against fish spoilage flora (Elotmani and Assobhei, 2004). 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). The combination of nisin and lactoferrin showed
increased antilisterial activity when tested in culture media (Branon and Davidson, 2004). Synergy between eukaryotic antimicrobial peptides and bacteriocins has also been investigated. The antimicrobial activity of pleurocidin (an antimicrobial peptide from fish) against E. coli was greatly enhanced by pediocin PA-1, sakacin P, and curvacin A (Lüders et al., 2003). Although no potential application has been proposed for the observed synergy, the available synthetic pleurocidin could probably be used in combination with bacteriocins for selective inhibition of Gram-negative bacteria in fish.

3.2. Bacteriocins and heat treatments

Bacteriocins can be used to reduce the intensity of heat treatments in foods without compromising microbial inactivation. Nisin and heat act synergistically against L. plantarum and L. monocytogenes (Mahadeo and Tatini, 1994; Ueckert et al., 1998), reducing the heat resistance of L. monocytogenes in milk (Maisnier-Patin et al., 1995) and in cold-pack lobster meat (Budu-Amoako et al., 1999). Nisin-resistant L. monocytogenes cells grown in the presence of nisin were more sensitive to heat at 55 °C than wild-type cells (Modi et al., 2000). The efficacy of enterocin AS-48 was higher on S. aureus cells sub-lethally injured by heat due to the lower concentration of remaining viable cells and to the cell damage induced by the heat treatment (Ananou et al., 2004). Bacteriocins can also provide an additional protection during food storage against proliferation of endospores surviving heat treatments. Moreover, it has been demonstrated that the intensity of heat treatments against bacterial endospores can be lowered in combination with nisin as well as with enterocin AS-48 (Beard et al., 1999; Wandling et al., 1999; Grande et al., 2006), saving costs in the heat treatment and decreasing the impact of heat on the food. Sub-lethal heat has been shown to sensitize Gram-negative bacteria to several bacteriocins such as nisin or pediocin ACh (Kalchayanand et al., 1992; Boziaris et al., 1998), enterocin AS-48 (Abriouel et al., 1998; Ananou et al., 2005), or jenseniin G (Bakes et al., 2004), extending their spectrum of action. Highest sensitization was reported for combined treatments of bacteriocins, heat and a chelating agent (Abriouel et al., 1998; Ananou et al., 2005).

3.3. Bacteriocins and modified atmosphere packaging

Modified atmosphere packaging (MAP) is frequently used in the food industry to prolong the shelf life of perishable food products. MAP may be defined as “the enclosure of food products in gas-barrier materials, in which the gaseous environment has been changed” (Young et al., 1988). Prolongation of the shelf life of food by MAP is based on retardation of intrinsic food changes and inhibition of spoilage microbiota. In a modified atmosphere, the dissolved CO₂ will determine growth inhibition of microorganisms (Devlieghere et al., 1998). Gram-negative bacteria are generally more sensitive to CO₂, while lactic acid bacteria are much more resistant (Farber, 1991; Church, 1994). Since Gram-negative bacteria are usually not sensitive to bacteriocins, MAP and bacteriocins are therefore two complementary hurdles of advantage to food spoilage.

Fang and Lin (1994a,b) found that growth of L. monocytogenes was completely inhibited on pork immersed in 10 IU/ml nisin and packed in 80% CO₂/20% air during 30 days of storage at 4 °C. Activity of nisin as well as ALTA™ 2341 against L. monocytogenes also increased in cold smoked salmon packaged under vacuum as well as under a 100% CO₂ atmosphere (Nilsson et al., 1997; Szabo and Cahill, 1999). A cocktail of seven L. monocytogenes isolates was inhibited by nisin as well as by ALTA™ 2341 under a 100% CO₂ atmosphere, but not under 100% N₂, or 40% CO₂/60% N₂ (Szabo and Cahill, 1998). It has been reported that nisin and CO₂ atmosphere acted synergistically on the cytoplasmic membrane of L. monocytogenes by enhancing membrane permeabilization (Nilsson et al., 2000).

3.4. Bacteriocins and pulsed electric fields

Pulsed electric field (PEF) technology is a non-thermal process where microbial inactivation is achieved by application of high-voltage pulses between a set of electrodes (Vega-Mercado et al., 1997). The effects of PEF resemble bacterial electroporation, but the higher intensity of this treatment causes severe damage to the bacterial cell membrane. Although this technology can only be applied to pumpable food products, it has gained attraction in recent years as an individual treatment or in combination with other hurdles such as bacteriocins. Since most bacteriocins act on the bacterial cytoplasmic membrane, the combined application of bacteriocins and PEF is expected to elicit increased bactericidal effects. Moreover, bacteriocins could also provide an additional hurdle against survivors from PEF treatments, such as sub-lethally injured cells or bacterial endospores (Fig. 3). PEF could also be applied to extend the antimicrobial spectrum of bacteriocins, since PEF disrupts the bacterial outer membrane allowing bacteriocin molecules to reach the bacterial cytoplasmic membrane target (Fig. 3). The efficacy of the combined treatments of PEF and bacteriocins in food preservation depends on several factors related to the PEF treatment (such as field strength, number of pulses, wave form or pulse duration), the food microbial load, composition and physiological stage, the added bacteriocin, and other environmental factors (Wouters et al., 2001; Bendicho et al., 2002; Heinz et al., 2002). All of these may have an influence on the numbers and types of bacteria surviving the combined treatment and, most important, their proliferation during the shelf life period of the processed food. For this reason, particular applications of bacteriocins and PEF must be studied in detail for each type of food and target bacteria.

Several investigations have thrown light on the effectiveness of combined treatments of bacteriocins and PEF treatments in food systems (Table 2). Exposure of L. innocua to nisin in liquid whole egg following PEF treatment exhibited an additive effect on inactivation of the microorganism (Calderón-Miranda et al., 1999a). A synergistic effect was observed as the electric field intensity, number of pulses and nisin concentration increased both in liquid whole egg and in skim milk (Calderón-Miranda et al., 1999a,b). L. innocua treated by PEF-nisin in skimmed milk exhibited an increase in the cell wall
roughness, cytoplasmic clumping, leakage of cellular material, and rupture of the cell walls and cell membranes (Calderón-Miranda et al., 1999c). It has been reported recently that the efficiency of the combined treatment of nisin and PEF in liquid whey protein concentrate was strongly dependent on the sequence of application, since exposure to nisin after PEF produced a lower effect on L. innocua inactivation. This behaviour was mainly attributed to changes in the cell envelope and to modifications of the medium caused by PEF application (Galvo et al., 2007).

The application of nisin clearly enhanced the lethal effect of PEF treatment on other Gram-positive bacteria such as Micrococcus luteus cells in phosphate buffer (Dutreux et al., 2000), L. plantarum in model beer (Ulmer et al., 2002), S. aureus in skim milk (Sobrino-Lopez and Martin Belloso, 2006), and vegetative cells of B. cereus (Pol et al., 2000). The synergy between nisin and PEF treatment against resting vegetative cells of B. cereus was enhanced by carvacrol (0.5 mM). Nisin showed less activity against B. cereus in milk compared to buffer (Pol et al., 2001a).

Bacterial endospores are refractile to PEF treatments, and incorporation of bacteriocins into the food may provide an additional hurdle against surviving endospores. Treatment of B. cereus spores with nisin and/or PEF treatment did not lead to direct inactivation of the spores or increased heat sensitivity as a result of sub-lethal damage. In contrast, germinating spores were found to be sensitive to PEF treatment. Nisin treatment was more efficient than PEF treatment for inactivating germinating spores (Pol et al., 2001b). Accordingly, bacteriocins could be applied as an additional hurdle against endospores surviving PEF treatment, provided that bacteriocin molecules remain active in the treated food.

Several studies have shown that the efficacy of PEF against Gram-negative bacteria can be enhanced by nisin. When PEF treatment was applied to Salmonella cells in orange juice in the presence of nisin (100 U/ml), lysozyme (2,400 U/ml), or a mixture of nisin (27.5 U/ml) and lysozyme (690 U/ml), cell viability loss was increased by an additional 0.04 to 2.75 log cycles. The combination of nisin and lysozyme had a more pronounced bactericidal effect (by at least 1.37 log cycles) than either nisin or lysozyme alone (Liang et al., 2002). Similarly, PEF treatment combined with cinnamon or nisin triggered cell death of E. coli O157:H7 in fresh apple cider, resulting in a reduction in viable counts of 6 to 8 log cycles (Iu et al., 2001).

Although nisin was totally inactivated by PEF treatment in simulated milk ultrafiltrate media, a 4-log cycle reduction of inoculated E. coli cells was accomplished with nisin (ca. 1,000 IU/ml) and three pulses of 11.25 kV/cm or 500 IU/ml for five pulses of the same intensity (Terebiznik et al., 2000). Nisin-PEF inactivation of E. coli in simulated milk ultrafiltrate media was enhanced by water activity reduction. Decreasing water activity to 0.95 with NaCl and applying PEF at 5 kV/cm (a non-lethal intensity when no other hurdle is used) with the further addition of nisin (1200 IU/ml) resulted in a 5-log cycle reduction of the bacterial population (Terebiznik et al., 2002). Nisin also increased PEF effectiveness against Pseudomonas aeruginosa.

At high PEF intensities (i.e., 11 kV/cm), the inhibitory effect of nisin increased with the number of pulses applied (Santi et al., 2003). In conclusion, addition of nisin can improve the efficacy of PEF treatments against vegetative cells of foodborne pathogens.
pathogenic and spoilage Gram-positive and Gram-negative bacteria. Presumably, similar effects are to be expected for other bacteriocins, although experimental data are needed to support this conclusion. The stability of bacteriocins to PEF treatments is another issue to be addressed, since remaining bacteriocin activity may provide an important hurdle against survivors (such as bacterial endospores) after PEF treatment.

3.5. Bacteriocins and high hydrostatic pressure (HHP)

High hydrostatic pressure (HHP) is an innovative food processing and preservation method that causes injury and killing of microbial cells (Kalchayanand et al., 1994; Farkas and Hoover, 2000; Patterson, 2000; Ray, 2002). A variety of pressure-treated products, such as ready-to-eat chicken meat, sliced ham, fresh whole oysters, jams, fruit juices, and guacamole, are now commercially available. During pressurization, the disruption of H-bonds, ionic bonds and hydrophobic interactions of the macromolecules adversely affects their structures and functions (Hoover, 1993). The sub-lethal damage is initiated by membrane phase transitions (Kato and Hayashi, 1999), affecting mainly ATP-generating and transport proteins. Cell death caused by HHP increases with pressure and so does the synergism with bacteriocins. Since most bacteriocins act on the bacterial cytoplasmic membrane it can be hypothesized that the observed synergy between bacteriocins and HHP results from cumulative damage to this structure. However, bacteriocin–membrane interaction during phase transition at high pressure has never been studied. A tailing effect is often observed after application of HHP treatments (Kalchayanand et al., 1998a), indicating that cell death occurs as a consequence of multiple events or cumulative cell damage. Sub-lethally injured vegetative cells surviving HHP treatment may develop pressure resistance, as has been previously reported for E. coli (García-Graells et al., 1998) and L. monocytogenes (Karatzas and Bennik, 2002). The increased cell damage caused by combined treatments of HHP and bacteriocins could prevent the tailing effect, providing an additional hurdle against selection of pressure-resistant vegetative cells.

The bactericidal effect of HHP (as well as its negative effects on food constituents) increases along with the temperature, which determines the different modalities of treatment, eg. cold HHP pasteurisation (ca. 5 °C), HPP-assisted pasteurisation (ca. 40 °C), or HPP-assisted sterilisation (ca. 90 °C). The food pH is also an influencing factor, and bacteria are usually more resistant to HHP in low acid foods. Addition of bacteriocins could improve the efficacy of HHP treatments in foods, compensating for the required increase in pressure or temperature.

The bacterial type and physiological stage (e.g. vegetative cells or endospores) may have great influence on HHP efficacy (Chen et al., 2006). Although bacteriocins are generally inactive on Gram-negative bacteria, HHP transiently sensitizes Gram-negative bacteria through outer membrane damage, increasing the possibilities for application of bacteriocins in food preservation (Kalchayanand et al., 1994; Hauben et al., 1996; Masschalck et al., 2001; Black et al., 2005). HHP also induces a more persistent sensitisation of Gram-negative bacteria to small diffusible antimicrobial molecules (García-Graells et al., 1998, 2000), which may act synergistically with other hurdles as well. Bacterial endospores are resistant to HHP treatments currently applied to foods (Smelt, 1998), although HHP treatments can induce endospore germination. Addition of bacteriocins as a second hurdle against surviving endospores could also improve the safety and shelf life of HHP-processed foods (Shearer et al., 2000).

Several studies have described the combined effects of bacteriocins and HHP on bacteria (Table 3). Nisin in combination with HHP showed strong synergistic effects against L. plantarum, E. coli and L. monocytogenes (ter Steeg et al., 1999; Farkas et al., 2003). HHP transiently sensitized Gram-negative bacteria to nisin, and a mechanism of pressure-promoted uptake of antimicrobial proteins and peptides was proposed to explain this sensitisation (Masschalck et al., 2001).

Pediocin AcH also increased decimal reductions caused by HHP on food spoilage and pathogenic bacteria in peptone solution (Kalchayanand et al., 1998b), and increased cell lysis in L. mesenteroides through cell wall degradation (Kalchayanand et al., 2002). The synergistic activity of pediocin AcH in combination with nisin was greatly enhanced during pressurization (Kalchayanand et al., 1998a,b, 2004a). A combination of nisin/pediocin AcH added to the plating medium was also effective on killing Clostridium spores induced to germinate by HHP treatment (Kalchayanand et al., 2004b).

A key issue in the efficacy of HHP treatments is the protective effect afforded by the food. For example, milk and meat (having a complex composition as well as a pH closer to neutrality) exert a greater protection against HHP. Inactivation of E. coli MG1655 was reduced from 7 logs at 400 MPa in phosphate buffer to only 3 logs at 700 MPa in milk, but addition of lysozyme (400 μg/ml) and nisin (400 IU/ml) to the milk increased the lethality of treatment (García-Graells et al., 1999). Combining HHP and nisin (500 IU/ml) also increased inactivation of bacteria associated with milk (Black et al., 2005). Similarly, the combination of lacticin 3147 and HHP increased the viability loss of S. aureus and L. monocytogenes in milk and in whey (Morgan et al., 2000), and residual lacticin still showed inhibitory effect in the food, inactivating and preventing growth of sub-lethally injured cells. Since HHP may have unwanted effects on milk components (Trujillo et al., 2002), bacteriocin-HHP treatments could serve to decrease the intensity of HHP treatment without compromising microbial inactivation.

Combined bacteriocin-HHP treatments have successfully applied for inhibition of foodborne pathogens in cheese. Survival of L. monocytogenes Scott A in cheeses made from raw milk that were previously inoculated with nisin and other bacteriocin-producer strains decreased as the intensity of HHP treatment increased (Arquiés et al., 2005). Nisin-HHP has also shown to increase inactivation of Bacillus and Clostridium endospores (Roberts and Hoover, 1996; Stewart et al., 2000). In Mato’ cheese, combining nisin with high pressure improved the biocidal effect on spores and aerobic mesophilic bacteria (Capellas et al., 2000). In model cheeses submitted to a germination cycle of 60 MPa at 30 °C for 210 min, followed by a vegetative cells destruction cycle of 300 or 400 MPa at
30 °C for 15 min the presence of nisin significantly increased inactivation of *B. cereus* spores and reduced proliferation of the surviving fraction measured 24 h and 15 d after HHP treatment (López-Pedemonte et al., 2003).

In liquid whole egg, HHP inactivation of *E. coli* and *L. innocua* improved significantly with nisin addition. A reduction of almost 5 log units in *E. coli* counts and more than 6 log units for *L. innocua* was reported at 450 MPa and 5 mg/l of nisin, and the two microorganisms were not detectable after one month of storage at 4 °C (Ponce et al., 1998).

The application of HHP to fresh meat products may result in a rubbery consistency (Murano et al., 2002). Bacteriocin addition could improve the efficacy of HHP treatments at lower pressure without adverse effects on meat (Hugas et al., 2002). In a meat model system, nisin reduced viable counts of *E. coli*, reduced growth of *S. aureus*, and suppressed slime-producing bacteria. Increased cell lysis through cell wall degradation in *L. mesenteroides* and *S. liquefaciens* was reported (Kalchayanand et al., 1998). Increased cell lysis through cell wall degradation in *L. mesenteroides* was reported (Kalchayanand et al., 2002).

**3.6. Bacteriocins and other non-thermal treatments**

In spite of the many other non-thermal treatments currently under study for food processing application, only a few reports have been published on their combination with bacteriocins. Irradiation offers a great potential for application in food preservation (Farkas, 1998, 2006). The spectrum of applications of food irradiation could be expanded in combination with bacteriocins, especially if the radiation dose can be lowered, since low-dose gamma irradiation has less unwanted effects on food and may also have better acceptance among consumers. It was reported that the combined application of pediocin (as ALTA™ 2341) and low-dose irradiation (2.3 kGy) had an increased antimicrobial effect on *L. monocytogenes* on frankfurters (Chen et al., 2004a).
One of the main limitations of gamma irradiation is the increased resistance of bacterial endospores (Farkas, 1998), requiring the application of combined treatments for a higher effectiveness. In sous vide meals inoculated with spores of psychrotrophic *B. cereus*, a combined treatment of nisin, heat (90 °C, 10 min) and gamma irradiation (5 kGy) markedly reduced the number of survivors for at least 42 days at an abuse temperature of 10 °C (Farkas et al., 2002).

Other non-thermal treatments such as pulsed magnetic fields (PMF) have also been tested in combination with bacteriocins. However, a combined treatment of PMF with EDTA and nisin had no effect on *E. coli* (San Martín et al., 2001).

**4. Bacteriocins and recent advances in molecular biology and genome studies**

Advances in molecular biology and molecular microbial ecology have provided new valuable tools to study microorganisms in food ecosystems, such as the determination of their bacteriocinogenic potential, the capacity for proliferation and inhibition of unwanted bacteria, or the response to stress factors. The distribution of bacteriocin-encoding genes among food isolates is a common issue that can be easily be resolved by molecular techniques. As an example, Maldonado et al. (2002) established that the plantaricin S operon is widely distributed among wild-type *L. plantarum* strains from olive fermentations by PCR amplification and hybridization with specific probes. Similarly, Ben Omar et al. (2004) analyzed the incidence of structural genes for several known bacteriocins among food isolates of *Enterococcus faecalis* and *E. faecium* by PCR amplification, and Fayé et al. (2004) studied the distribution of propionicin T1 genes among propionibacteria in a similar way. PCR amplification with specific primers for bacteriocin genes may be used to follow the predominance of an inoculated strain in food fermentation, as shown for sakacin-P producing *L. sakei* during production of fermented sausages (Urso et al., 2006a) and *L. gasseri* K7 in semi-hard cheese (Matijašie et al., 2007). Similarly, multiplex PCR targeting both bacteriocin genes and species-specific genes can serve for precise identification of bacteriocinogenic strains in a single analysis. Nisin-producing lacticocci and diercvin 41 producing *C. divergens* V41 were identified rapidly by this procedure (Moschetti et al., 2001; Connil et al., 2002). Multiplex PCR was also used successfully to follow implantation of V41 strain in cold smoked salmon (Connil et al., 2002). Other procedures such as analysis of RAPD-PCR profiles (Ryan et al., 2001; Matijašie et al., 2007), REP-PCR (Foulquié-Moreno M.R., Verluyten J., Vancanneyt M., Adriany T., Leroy F., Swings J., and De Vuyst L., unpublished) or PFGE analysis (Moschetti et al., 2001) have also been used to evaluate strain implantation in food systems and the impact of bacteriocinogenic starters on the food quality from the microbiological point of view. A recent study addressed the use of DGGE to evaluate simultaneously the impact of a bacteriocinogenic LAB on foodborne pathogens inoculated in a fermented food as well as on the overall microbiological profile of the fermentation (Díaz G., Ben Omar N., Abriouel H., Lucas R., Martínez Cañamero M., and Galvez A, unpublished).

Real-time PCR could be used to follow survival of target bacteria in the presence of added or in situ produced bacteriocin while simultaneously determining the growth of bacteriocinogenic strains. This approach could provide additional data independent of the biases introduced by sample preparation procedures and the pressure of selective media currently used for enumeration of microorganisms. In other cases, it could solve the problems of differentiation of closely related bacteria in mixed populations. Grattepanche et al. (2005) used this method to evaluate the impact of the nisin Z-producing *L. lactis* subsp. *lactis* biovar. *diacetylactis* strain UL719 on *Lactococcus cremoris* in milk fermented with mixed cultures, avoiding the problem to distinguish the two bacterial populations in different media when they were in the same order of magnitude. Real-time PCR could also provide more precise information on cells sub-lethally injured by bacteriocin molecules. Similarly, DNA-based technology could be used to follow the expression of bacteriocin genes in food systems, as well as the influence of environmental conditions on gene expression and the stress response of target bacteria to the produced or added bacteriocin in food. In a recent work, expression of sakacin P structural gene *sspA* by the *L. sakei* strain 1151 in sausages was studied in order to determine the influence of the production procedure for fermented sausages on bacteriocin production (Urso et al., 2006b). This alternative method could overcome the biases introduced by the currently used extraction procedures on the determination of the amounts of bacteriocin produced in the food.

The use of fluorescence-based technology (such as fluorescence *in situ* hybridisation—FISH—, confocal laser microscopy, or fluorescence ratio imaging microscopy—FRIM—) could also provide valuable information, for example, on the distribution of bacteriocinogenic strains within the food matrix (Fernández de Palencia et al., 2004) or the heterogeneous response of bacterial populations to bacteriocins (Hombæk et al., 2006). Other suggested applications could be to study the distribution of target bacteria in the food both in the absence and in the presence of bacteriocin pressure and the generation of gradients and protected niches as a function of bacteriocin concentration. These techniques may facilitate the study of the effects of bacteriocins in food systems at the level of single cells, providing a completely new scenario picture of bacteriocin effects.

Another line of research on bacteriocin application may rise from genomic studies. Classical methods for detection of produced bacteriocins may underestimate the bacteriocinogenic potential of LAB due to several factors such as the influence of environmental conditions on bacteriocin production, the inducible character of many bacteriocins, and the loss of the production capacity (which may be caused by gene mutation, gene loss or genetic rearrangements). However, the analysis of complete genomes may reveal the presence of potential bacteriocin genes and new bacteriocins independently of the producer capacity of strains (Nes and Johnsborg, 2004). As an example, in silico analyses of the probiotic strain *Lactobacillus acidophilus* NCFM predicted a chromosomal locus for lactacin B, a class II bacteriocin (Altermann et al., 2005). Similarly, while a limited number of bacteriocins have been described in
Streptococcus thermophilus (Gilbreth and Somkutti, 2005 and references cited therein), analysis of the genome sequences available revealed two loci predicted to be related to bacteriocin production (Hols et al., 2005). The first locus (lab, for lantibiotic) contains genes that are similar to genes generally found in lantibiotic production loci. However, it is not clear whether this locus is really involved in lantibiotic production. The second locus is similar to the bacteriocin-like peptide (blp) locus described in S. pneumoniae and displays the typical characteristics of a Class II bacteriocin locus. The blp loci of the S. thermophilus strains also contain genes encoding bacteriocin-like peptides (i.e., lpD, blpU, blpE, blpF in strain LMD9, blpU, blpK in strain LMG18311, and blpK in strain CNRZ1066 (Hols et al., 2005). However, nonsense mutations detected in some of the regulatory genes indicate that this locus is not functional at least in some strains. The data available suggest that bacteriocin loci in S. thermophilus could be exploited by genetic engineering in order to develop new bacteriocinogenic strains of technological interest. Similarly, genome sequencing of L. sakei 23 K has revealed that this strain carries a 6.5 kb region containing part of the spp gene cluster responsible for sakacin P production (Morett et al., 2005) described previously in L. sakei LB674 (Hühne et al., 1996). Since spp homologue genes (mainly sppK and sppR homologues) seem to be widely distributed among L. sakei, it has been suggested that these genes could be used as a fingerprinting region in typing methods and/or as a marker for L. sakei strains (Morett et al., 2005).

Many bacteriocins are encoded by small genes that are often omitted in the annotation process of bacterial genomes (De Jong et al., 2006). In addition, bacteriocins and their accessory proteins are often encoded by poorly conserved ORFs. The identification of genes that are functionally similar but have limited or no sequence homology is often a problem in genome data mining. A web server (BAGEL) that identifies putative bacteriocin ORFs in a DNA sequence using novel, knowledge-based bacteriocin databases and motif databases has been recently created (De Jong et al., 2006). BAGEL is freely accessible at: http://bioinformatics.biol.rug.nl/websoftware/bagel. Hopefully, this software will help researchers to identify novel bacteriocin-related genes in the upcoming LAB genome sequences.

A recent data mining study demonstrated that the genome of Pediococcus pentosaceus ATCC 25745 contains a gene cluster that resembles a regulated bacteriocin system (Diep et al., 2006). The gene cluster has an operon-like structure consisting of a putative pediocin-like bacteriocin gene (termed penA), a potential immunity gene (termed peiA) as well as genetic determinants involved in bacteriocin transport and regulation. Nevertheless, the accessory gene involved in transport and the inducer gene involved in regulation are missing, which makes this bacterium a poor bacteriocin-producer. Cloning of penA and peiA in a L. sakei host that contains the complete apparatus for gene activation, maturation and externalization of bacteriocins confirmed the production of this new and potent bacteriocin, termed penocin A (Diep et al., 2006).

Based on the genome sequences and gene identification, it will be possible to develop adequate methodologies (such as microarray technology) to study the global response of bacteria to bacteriocins. In one example, by using microarrays for global analysis of gene expression, it has been shown that lactococcin 972 induces a cell-envelope stress response in L. lactis mediated by the two-component system KinD/LlrD (Martínez et al., 2006). Transcriptome analysis can also reveal new and relevant data on the biology of bacteriocins such as the mechanisms of resistance/adaptation. Strains of L. lactis that are naturally adapted or transiently resistant to nisin can be readily obtained by subcultivation with sub-lethal nisin amounts. The adaptation is lost upon subcultivation without nisin. Transcriptome analysis of adapted strains revealed a significant up/down regulation of 95 genes belonging to several main functional categories: (i) cell wall synthesis, (ii) central and energy metabolism, (iii) phospholipid- and fatty acid metabolism, (iv) gene regulation, (v) transport, (vi) stress, and (vii) miscellaneous or unknown functions (Kok et al., 2005). The comparative transcriptome analysis of nisin-sensitive and nisin-resistant L. lactis concluded that nisin resistance is a complex phenotype, involving various different mechanisms, mainly (i) preventing nisin from reaching the cytoplasmic membrane, (ii) reducing the acidity of the extracellular medium, thereby stimulating the binding of nisin to the cell wall, (iii) preventing the insertion of nisin into the membrane, and (iv) possibly transporting nisin across the membrane or extruding nisin out of the membrane (Kramer et al., 2006). Studies similar to these are clearly needed in order to elucidate the mechanisms that may be involved in adaptation of foodborne pathogens upon exposure to bacteriocin pressure in food systems.

Overall, the set of methodologies that have emerged in recent years provide an arsenal of barely unexplored tools that could expand the potential of bacteriocinogenic strains for food application and improve our understanding on the global effects of bacteriocins in food ecosystems, allowing a more rational application of these natural antimicrobial hurdles in foods.

5. Conclusions

A large number of bacteriocins from LAB have been characterized to date, and many different studies have indicated the potential usefulness of bacteriocins in food preservation. Bacteriocins are a diverse group of antimicrobial proteins/peptides, and therefore are expected to behave differently on different target bacteria and under different environmental conditions. Since the efficacy of bacteriocins in foods is dictated by environmental factors, there is a need to determine more precisely the most effective conditions for application of each particular bacteriocin. The use of novel preservation technologies offers new opportunities for application of bacteriocins as part of hurdle technology, as has been demonstrated for PEF and HHP. However, the combined application of many other technologies (such as ultrasanication, irradiation, microwave and ohmic heating, or pulsed light) still remains unexplored. Bacteriocinogenic cells may also act as living factories in foods. The antimicrobial effects of bacteriocins and bacteriocinogenic cultures in food ecosystems must be understood in terms of microbial interactions. The application of a microbial ecology approach may provide a more realistic portrait of the complex interactions.
occurring in food systems that ultimately lead to microbial inactivation, survival or adaptation to environmental stress. The use of molecular microbial ecology methodologies may help to understand better the biology of bacteriocins and food microbial ecosystems at cellular and molecular levels. The study of bacterial genomes and other related aspects such as global gene expression analysis can provide valuable information on the bacteriocinogenic potential of LAB. Genomic studies can also throw light on other issues of great interest in food application such as the global response of ecosystems to bacteriocins or the development of adaptation or resistance.

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