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Review

Natural antimicrobial proteins: a review of current challenges and solutions for food applications

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Abstract: There has been growing interest in the use of antimicrobial proteins – lactoferrin, lysozyme, nisin, lactoperoxidase systems and their derivatives – as an alternative of non-thermal food preservation methods. However, the potential of these proteins in food suffers from the major disadvantage of their being intercepted by other food components that could interact with the proteins themselves, the oxidation products or with their targets, the extent of which depends on the protein modes of action and chemical composition of the food. To ensure their effectiveness in or on food, the proteins are immobilized inside microcapsules or in the matrix of polymers, or used in combination with other preservatives and treatments.

Introduction

A recent trend in food preservation is directed towards employing minimal additives and thermal treatment, but with assured microbial safety and shelf life stability to comply with consumers' demands for natural and healthy food. Such demands have led to the exploitation of non-thermal preservation methods to control undesirable microorganisms while retaining both nutritional and sensory properties of the food. The use of natural preservatives is viewed as an appropriate alternative because they are readily available in plants, animals, insects, and microorganisms where they evolve as part of their hosts' defense mechanisms against microbial invasion [1, 2].

Of the antimicrobial systems present in nature, antimicrobial proteins (AMPs) have captured much attention for use as food preservatives and they have recently been the subject of extensive reviews [3, 4, 5, 6]. This interest may be due to several reasons: (i) AMPs, as proteins, can be easily digested in the stomach so as to not lead to residue build-up and some of them (e.g., lactoferrin and other bioactive peptides) may even contribute to health benefits, including prevention and treatment of disease; (ii) AMPs tend to produce less undesirable effects on the sensory characteristics of food, compared with other antimicrobial systems such as phenolics or oils [1]; and (iii) AMPs show rapid killing action, highly selective toxicity and low potential for development of resistance [7].

Hitherto, numerous AMPs have been discovered in nature, yet only a few of them have been commercially utilized in food. The potential applications of other AMPs may be hampered by two main factors. On one hand, there is a need to conduct extensive research on the extraction, isolation, safety, efficacy, application methods and product quality influences before they could be used in food systems [1]. On the other hand, an AMP must function in a food system and its spectrum of action should be as broad as possible to be economically used. Although numerous AMPs have been found to have potential activity in laboratory media, their effectiveness in food has not been investigated thoroughly. Results derived from experiments *in vitro* with laboratory media do not always signify their potential in food, which is made up of complex systems and possesses many variables such as temperature, atmosphere, pH, oxidation-reduction potential, water activity and other factors that may interfere with the antimicrobial activity.

Most of the previous reviews dealing with AMPs focused mainly on production, mechanisms of action, possible applications or strategies designed to broaden their spectra of action. A current challenge of using antimicrobials for food preservation, however, is the significant reductions of their efficacies due to interactions of the antimicrobials with other components present in food systems [8]. Indeed, an understanding of such interactions would be of practical importance when antimicrobials are developed or selected for use in food preservation. To this end, the current review will present an overview of AMPs for food preservation, with a particular emphasis on recent findings of their mechanisms of action; discuss factors limiting their effectiveness in foods; and present some useful approaches that have been developed to effectively use the antimicrobials in or on food.

Overview of AMPs for Food Preservation

For ease of discussion, AMPs will be divided into five classes on the basis of the protein characteristics.

Metal-ion binding proteins

The best characterized metal-ion binding proteins include lactoferrin from milk [9], ovotransferrin [10] and phosvitin from eggs [11]. The proteins have long been known to exert their antimicrobial effects through depriving microorganisms of multivalent metals, namely Fe, Mg, and Ca. These three ions play essential roles in the growth and survival of most microorganisms. Iron is used for the growth, replication, DNA synthesis and respiration of aerobic and facultative anaerobic species. Magnesium is required for the activity of many membrane-bound enzymes and it also acts as a salt-bridge for the supramolecular structure in the lipopolysaccharides (LPS) of gram-negative bacteria. Calcium also has a role in stabilizing LPS and regulating cellular processes involving nucleoid structure, protein phosphorylation and alterations in transverse and lateral distributions of membrane lipids [12]. Therefore, once the ions are limited or deprived, susceptible organisms may not be able to grow and/or lose viability [13]. In spite of this, there is also some experimental evidence that some, but not all, of the proteins in this class still retain their antimicrobial activities even after being saturated with metal ions, suggesting that direct interactions between the protein molecules, presumably due to their cationic properties, with cell membrane components (including LPS) may also be implicated with their antimicrobial effects [3, 14].

Hydrolytic proteins

Lysozymes, chitinases and β-glucanases are typical of hydrolytic proteins, which are known to operate the antimicrobial action by degrading key structural components of the cell walls of bacteria and/or fungi [15]. Hen egg lysozyme (Lz) has particularly been extensively investigated for food and pharmaceutical applications. This interest is not because Lz is a superior antimicrobial protein, but because (i) its steric structure has been clearly defined; (ii) it is relatively inexpensive due to its abundance in egg white (up to 3.5% of dry weight); and (iii), being a very basic protein, it can be modified by allowing it to interact with other components in efforts to improve its antimicrobial spectrum of action.

The mechanism by which Lz kills sensitive organisms has long been attributed to the degradation of the 1,4 β-D-linkage between N-acetylhexosamines in the peptidoglycan layer of bacterial cell wall. However, this lytic mechanism has recently been challenged by a number of investigators. Evidence has accumulated that Lz can also kill its susceptible bacteria by membrane disruption; self-promoted uptake; and induction of autolysins, bacteriolytic enzymes that digest the cell wall peptidoglycan of the bacteria that produce them. These findings suggest that the mechanism(s) of action of Lz may vary with organisms, experimental conditions as well as conformational changes after its molecule has been modified [5, 6, 16].

Regardless of the mechanisms involved, it is generally accepted that Lz is predominantly sensitive to gram-positive bacteria. Notwithstanding having the ability to interact with LPS of the outer membrane, Lz has little effect on the overall cellular uptake and the viability of gram-negative bacteria [17], because the porin channels, a specific class of major proteins in planar lipid membranes through which Lz is postulated to enter the outer membrane en route to the periplasm, is only permeable to small molecular weights of up to 900–1000 Da [18]. Accessibility of Lz to its target sites can be enhanced by three main approaches: (i) disruption of the outer membrane, achieved either by physical or chemical treatments; (ii) increasing the surface hydrophobicity of Lz by genetic, physical or chemical means; and (iii) specific hydrolysis of the Lz molecule to yield more potent antimicrobial peptides [5, 6, 19].

Bacteriocins

Bacteriocins are proteinaceous compounds produced by bacteria and are lethal to other bacteria rather than the producing strains [20]. Of the bacteriocins identified to date, only nisin, produced by *Lactococcus lactis*, and related compounds such as pediocin are currently authorized for use as food preservatives [2]. Nisin is a 34 residue antibacterial protein with the presence of lanthionines and uncommon amino acids. Nisin acts on vegetative bacteria by a four-step process of binding, insertion, aggregation and pore formation. Nisin binds to the target membrane by electrostatic interactions with the anionic phospholipid and inserts into the membrane by its hydrophobic patches [21]. Nisin, however, acts on the sulfhydryl membrane groups for inactivation of germinated spores [22]. As is the case with Lz, nisin is known to be reactive to gram-positive bacteria and spores while being resistant to gram-negative bacteria and yeast or filamentous fungi.

Antimicrobial peptides

With few exceptions, antimicrobial peptides are small molecules with a molecular mass ranging from 1 to 5 kDa [20]. There are two major types of antimicrobial peptides: cationic and bioactive peptides. The cationic peptides are gene-encoded peptides composed of 12–50 amino acids, with at least two excess positive charges contributed by lysine and arginine residues and about 50% hydrophobic amino acids. Though they are ubiquitously available in nature, only magainins, defensins, cecropins, melittin, alamethicin and protamine are the most well-known and studied. These peptides have broad spectra of activity, being able to kill or neutralize gram-negative and gram-positive bacteria, fungi, yeasts, parasites, viruses and cancer cells. They normally kill their target organisms by initial electrostatic interactions with the negatively charged surface of microbial cells, followed by self-promoted uptake to the cytoplasmic membrane [7]. The peptides have been studied mainly for use as therapeutic agents; their potential as food preservatives may be restricted due to their high production costs [23].

The bioactive peptides, usually composed of 3–20 amino acid residues per mole [24], are produced by hydrolysis of food proteins: caseins, α-lactalbumin, β-lactoglobulin, lactoferrin and lysozyme. Interestingly, these peptides exhibit stronger antimicrobial activity than their parent molecules and are currently being investigated for use in

health-promoting food. Perhaps the most powerful one is the peptide derivative from the N-terminal part of bovine lactoferrin called lactoferricin, whose activity is up to one hundred times more potent than that of the parent molecule. This strongly basic antimicrobial peptide is 23 amino acids long, which is different from the iron-binding region. Cationic nature and helix propensity play critical roles for interacting with the microbial cell surface and destabilizing the cytoplasmic membrane [25]. The peptide has broad spectra of activity against gram-negative bacteria, gram-positive bacteria, fungi, protozoa and viruses *in vitro* and also possesses other biological properties, including antioxidant, anti-cancer and anti-inflammatory activities [3].

Antimicrobial oxidoreductase systems

Oxidoreductases, which have no antimicrobial activity themselves, exert their antimicrobial effects by the generation *in situ* of toxic intermediary oxidation products [15]. This class includes glucose oxidase, xanthine oxidase, lactoperoxidase, myeloperoxidase and horseradish peroxidase, but so far only glucose oxidase and lactoperoxidase systems have been subject to extensive investigations for food preservation.

In the presence of O_2, glucose oxidase (GOD) catalyzes the breakdown of glucose to gluconic acid and the concomitant reduction of molecular oxygen to H_2O_2. Several properties enable GOD to be a useful preservative: (i) the enzyme can itself act as an O_2-scavenger and thus slow down enzyme-catalyzed discolouration reactions in packaged or canned food; (ii) the gluconic acid can act as metal-ion chelator; and (iii) the hydrogen peroxide by-product is a powerful oxidant which can cause damage to nucleic acids, proteins and lipids of microorganisms [26, 27]. The sensitivity of the system to microorganisms is determined by the absence or the presence of catalase and/or small compounds such as glutathione and ascorbic acid [15].

Lactoperoxidase (LPO), naturally present in milk, saliva and tears, is capable of catalyzing the oxidation of SCN^- and I^- at the expense of H_2O_2 to generate such molecules as hypothiocyanite ($OSCN^-$), hypoiodide (OI^-) or a mixture of both. The generated oxidation products exert antimicrobial effects against a wide range of bacteria, fungi and viruses by the oxidation of thiol groups (-SH) of cytoplasmic enzymes and damage to other cellular functions essential for microbial metabolism [28].

While the effects of the LPO–H_2O_2–I_2 system are generally bactericidal, there is still some controversy about the bacteriostatic and bactericidal effects of the LPO–H_2O_2–SCN system on gram-positive bacteria and gram-negative bacteria [29]. In fact, the efficacy of the latter system is largely influenced by pH, temperature, test medium and the ratios of the oxidation products per target cell [30].

Factors Limiting the Effectiveness of AMPs in Food

In many instances, the effects of AMPs have been reported to be much reduced in real food systems, compared to those observed with laboratory media (*in vitro*) and the

extent of reductions has been observed to be largely dependent on the complexity and/or chemical composition of the particular food to which they are added [9, 30, 31, 32, 33, 34, 35, 36]. There are many factors that may affect their potential in food systems, the most important of which is the interference of food components with the antimicrobials. Unlike the interactions *in vitro*, where the antimicrobials and organisms are equally in contact, the interactions in food involve a third factor, which is the food substrate. Food components such as other proteins, lipids, salts, thiols and proteases may impair the antimicrobial activity by interacting with the antimicrobial proteins themselves, the oxidation products or with their targets, the extent of which may depend on the type of proteins and their modes of action.

Food proteins, particularly those with negatively charged properties (e.g. bovine serum albumin and caseins or the micelles) and lipids (including, emulsifiers, fatty acids, phospholipids and milkfat) have been shown to reduce the activities of lysozyme, bacteriocins and antimicrobial peptides by intercepting the electrostatic and hydrophobic interactions between the antimicrobials and microbial cells and/or by preventing the organisms from being attacked [23, 37, 38, 39].

The existence of metal ions such as Na^+, K^+, Fe^{2+}, Ca^{2+} and Mg^{2+} in food is well established, with higher concentrations being generally found in food of animal origin than in fruit and vegetable products [40]. At levels present in food, these cations, particularly the divalent ones, may reduce the antimicrobial effects of the cell wall- and membrane-disrupting proteins by binding to the anionic cell wall components (LPS, phospholipids and porins), consequently neutralizing the negative charges of the microbial cell surface [41], condensing or tightening the membrane lipids [39] and blocking the porin and nonporin pathways [18]. In the case of the metal-ion binding proteins, the divalent cations may compete with the bound divalent cations in the outer membrane or replace the ones removed by the antimicrobials [9]. It is also noteworthy that heme-containing proteins (e.g., haemoglobin) in food where they are available can be sources for microorganisms to utilize in an iron-limited environment.

Thiol compounds such as glutathione, cysteine and sodium sulphite are effective in inhibiting the antimicrobial activities of nisin, GOD and LPO systems, either by direct binding to the haem group/dehydro residues of the proteins or by scavenging the oxidation products. Glutathione is particularly widespread in nature, being found in both plant and animal tissues and it could be one of the reasons why nisin and the LPO systems are poorly active in fresh meat products [32, 42]. However, it remains to be confirmed whether or not their antimicrobial activities could be affected by other food proteins containing free SH groups. de Wit & van Hooydonk [43] observed that SH groups of milk proteins such as β-lactoglobulin had no inhibitory effects on the LPO–H_2O_2–SCN system, but the antimicrobial effects of the system was found to be remarkably decreased in the presence of ovalbumin, a protein containing free SH groups in eggs [30].

Last but not least, the effects of proteases, though less obvious, may also be damaging to the activity of AMPs as this group of enzymes, originating from plant or animal tissues or from microorganisms, can degrade the antimicrobial molecules. The case is particularly more obvious with the bacteriocins and antimicrobial peptides [7, 36].

As a consequence of their interactions with food components, AMPs tend to be less available to interact with microbial cells. As such, the less available antimicrobials may produce only transient inhibition to the target organisms and regrowth to levels equivalent to those of untreated cells often occurs under appropriate conditions, especially when low or practical antimicrobial dosages are used [44, 45, 46]. The observations may support the notion that the exposed, sub-lethally injured cells are able to repair and resuscitate to the healthy state under appropriate conditions, for example at abused temperatures, while some cells (i.e. survivors) may develop resistance responses to the antimicrobials. Their responses range from the induction of specific sets of compounds–proteolytic enzymes to degrade the protein molecules [36, 47], catalase to decompose H_2O_2 [27], or small molecular-weight iron-binding compounds known as siderophores to utilize the iron sequestered by the proteins [10] –to changes in cell wall constituents [2], or the membrane lipids [48, 49].

Approaches to Enhancing the Potential of AMPs in Food Applications

AMP-chelator combinations

The effectiveness of AMPs in food, to some extent, can be enhanced when they are used in conjunction with chelating agents such as ethylenediaminetetraacetic acid (EDTA), polyphosphate, acetate, citrate, lactate, or the metal-ion binding proteins, as discussed above [9, 13, 37, 40, 50]. Advantageously, the addition of chelators into food could:

- help counteract the antagonistic effect of the divalent cations (Ca^{2+} and Mg^{2+}) on the activity of AMPs;
- help destabilize the outer membrane of gram-negative bacteria by either chelating or displacing the divalent cations and thus enhance the accessibility of AMPs (nisin and lysozyme) to reach their target sites [50];
- inhibit the activity of proteases that might degrade the antimicrobial molecules [47]; and prevent flavour, oxidative rancidity and colour changes in food through formation of complexes with free metal ions.

Again, chelating agents, however, are effective in culture media or less complex food systems such as fruit and vegetable products, but appear to have minimal effects in complex food systems (e.g., milk or meat products) where substantially high concentrations of the metal ions are often present [9, 31, 35, 37, 51]. In this context, unrealistically high levels of the chelators are needed to be effective in complex food; however, such treated products may not be accepted by consumers because of an adverse effect on the nutritional value of the food. The chemical chelators, on the other hand, have their own limitations with respect to applicability in food [50].

Multitarget attack/integrative approaches

It is now well-established that an integration of several hurdles in the preservation of a particular food is a promising approach due to the fact that different hurdles may simultaneously, if not synergistically, act on different targets (e.g., cell membrane, DNA, enzyme system and other cellular functions) within the microbial cells so that survival and cell repair would become more difficult [52]. Such concepts may be achieved by combining the effects of two or more antimicrobials, or with other preservatives and treatments.

The use of two or more antimicrobial proteins and/or mix with other preservatives (with or without chelating agents) to yield a synergistic effect on the target organisms has been extensively investigated as a means to offer many opportunities for practical exploitation of AMPs in food [9, 37, 44, 53, 54]. Practically, this synergism not only allows very low doses of the proteins to be used effectively, but also expands the range of organisms that may be inhibited by the proteins; that is, organisms normally resistant to each component of the mixture when used separately can also be usefully controlled [52].

Another approach, which has recently received growing interest from the food industry and researchers, is the use of AMPs with other non-thermal preservation methods, including high hydrostatic pressure, pulse electric fields, ultrasonication and irradiation as a potential pathogen intervention strategy for various food products. AMPs have been shown to act in synergy with these treatments [29, 55, 56]. The synergistic action of such combination preservation systems may offer several advantages: (i) improvement of the rate of inactivation; (ii) development of cost-effective mild preservation; and (iii) reduction of the commercial problems associated with sub-lethal injury and survivor tails [57].

Similarly, it is now being realized that the effects of AMPs are pronounced when combined with certain physical processes (i.e., mild heating, chilling, freezing, drying or homogenization) because microbial cells sub-lethally injured by the treatments may become more susceptible to the proteins to which the healthy cells are resistant [58, 59, 60, 61]. This approach may be of economical consideration to the food industry, not only because of the better image of the product, but also because of the reduced processing costs. An interesting instance of such treatments is the activation of the LPO systems in milk either before or after heat treatment to reduce the *D* values and to increase the keeping quality of the product [58, 60].

Microencapsulation approaches

Several attempts have been made to trap AMPs in multilamellar liposomes or vesicles to prevent the antimicrobial activity from inhibitors or unfavourable conditions in the food matrix [62, 63, 64]. Prepared under defined conditions, liposomes may retain both structural integrity and the contents after long storage; release the trapped proteins at the intended target site rather than being nonspecifically dispersed throughout the food matrix; and reduce the accessibility of the proteins to unfavourable conditions or elements in food systems [62, 63]. For example, Benech *et al*. [63] demonstrated that liposome-entrapped nisin retained higher activity than the non-immobilised protein

against *Listeria innocua* in the cheese matrix during ripening. The efficacy of the systems may, nevertheless, depend on liposome type, size, lipid composition, membrane fluidity, stability, charge, ease of preparations, temperature, pH, the permeability of the liposomal bilayer to substrate and properties of the molecular weight and size of the proteins to be encapsulated [64, 65].

Incorporation into polymers

Another interesting solution to ensure the potential of AMPs in food applications might involve the incorporation of the antimicrobials into either synthetic or edible polymer films [66, 67, 68, 69, 70, 71]. Comprehensive reviews of bioactive or antimicrobial food packaging have recently been published [72]. Indeed, this approach might be particularly applicable to food where the growth of organisms occurs primarily at the surface (e.g., meat and poultry products). Reportedly the approach has advantages over antimicrobial dips or sprays in terms of protein concentrations used and activity loss due to interactions with food components/surfaces [72]. In particular, when allowed to be immobilized to edible films and coatings prepared from polymer gels, AMPs have been shown to be able to sustain the antimicrobial activity during the storage period; localize at the site of bacterial growth; and reduce exposure to inactivating agents on the meat surface [66, 67]. By way of example, immobilized lactoferrin to glycosaminoglycan gels, so-called activated lactoferrin, has proven to be an effective system and already approved to be used to reduce microbial contamination on the surface of beef carcasses in the USA [70].

Conclusion

AMPs could be an alternative method for extending the shelf life of food. However, they would be best utilized for surface treatments or edible coatings and films because, when added as food ingredients, the antimicrobial activity is highly vulnerable to inactivation by other components or conditions within the food systems. If they are to be added to the bulk of the food, appropriate strategies must be designed to ensure their effectiveness. Factors such as mechanisms of action, chemical composition of the food, the type of microorganisms and the type and intensity of any further treatment (e.g., thermal or non-thermal treatments) need to be taken into consideration to select suitable antimicrobials for a particular food. The future potential applications of AMPs can be substantial, particularly if they are used in a synergistic combination with other treatments, including packaging systems, to provide multi-hurdle preservation systems to extend the safe shelf life of food.

References

1. Gould, G.W. (1996). Industry perspectives on the use of natural antimicrobials and inhibitors for food applications. **Journal of Food Protection** (supplement), 82–86.

2. Brul, S. & Coote, P. (1999). Preservative agents in food. Mode of action and microbial resistance mechanisms. **International Journal of Food Microbiology**, 50, 1–17.

3. Shimazaki, K.I. (2000). Lactoferrin: a marvelous protein in milk? **Animal Science Journal**, 71, 329–347.

4. Kussendrager, K.D. & van Hooidonk, A.C.M. (2000). Lactoperoxidase: physico-chemical properties, occurrence, mechanism of action and applications. **British Journal of Nutrition**, 84, suppl.1, S19–S25.

5. Ibrahim, H.R., Aoki, T. & Pellegrini, A. (2002). Strategies for new antimicrobial proteins and peptides: lysozyme and aprotinin as model molecules. **Current Pharmaceutical Design**, 8, 671–693.

6. Masschalck, B. & Michiels, C.W. (2003). Antimicrobial properties of lysozyme in relation to foodborne vegetative bacteria. **Critical Reviews in Microbiology**, 29, 191–214.

7. Hancock, R.E.W. & Scott, M. G. (2000). The role of antimicrobial peptides in animal defenses. **Proceedings of the National Academy of Sciences of the United States of America**, 97, 8856–8861.

8. Devlieghere, F., Vermeiren, L. & Debevere, J. (2004). New preservation technologies: Possibilities and limitations. **International Dairy Journal**, 14, 273–285.

9. Branen, J.K. & Davidson, P.M. (2004). Enhancement of nisin, lysozyme and monolaurin antimicrobial activities by ethylenediaminetetraacetic acid and lacfoferrin. **International Journal of Food Microbiology**, 90, 63–74.

10. Baron, F., Gautier, M. & Brule, G. (1999). Rapid growth of *Salmonella enteritidis* in egg white reconstituted from industrial egg white powder. **Journal of Food Protection**, 62, 585–591.

11. Khan, M. A. S., Nakamura, S., Ogawa, M., Akita, E., Azakami, H. & Kato, A. (2000). Bactericidal action of egg yolk phosvitin against *Escherichia coli* under thermal stress. **Journal of Agricultural and Food Chemistry**, 48, 1503–1506.

12. Norris, V., Grant, S., Freestone, P., Canvin, J., Sheikh, F.N., Toth, I., Trinei, M., Modha, K. & Norman, R.I. (1996). Calcium signaling in bacteria. **Journal of Bacteriology**, 178, 3677–3682.

13. Vaara, M. (1992). Agents that increase the permeability of the outer membrane. **Microbiology Review**, 56, 395–411.

14. Valenti, P., Visca, P., Antonini, G. & Orsi, N. (1985). Antifungal activity of ovotransferrin towards genus candida. **Mycopathologia**, 89, 169–175.

15. Fuglsang, C.C., Johansen, C., Christgau, S. & Adler-Nissen, J. (1995). Antimicrobial enzymes: application and future potential in the food industry. **Trends in Food Science & Technology**, 6, 390–396.

16. Touch, V., Hayakawa, S., Fukada, K., Aratani, Y. & Sun, Y. (2003). Preparation of antimicrobial reduced lysozyme compatible in food applications. **Journal of Agricultural and Food Chemistry**, 51, 5154–5156.

17. Ohno, N. & Morrison, D.C. (1989). Lipopolysaccharide interaction with lysozyme. **Journal of Biological Chemistry**, 264, 4434–4441.

18. Witholt, B., van Heerikhuizen, H. & de Leij. (1976). How does lysozyme penetrate through the bacterial outer membrane. **Biochemistry Biophysica Acta**, 443, 534–544.

19. Touch, V., Hayakawa, S. & Saitoh, S. (2004). Relationships between conformational changes and antimicrobial activity of lysozyme upon reduction of its disulfide bonds. **Food Chemistry**, 84, 421–428.

20. Joerger, R.D. (2003). Alternatives to antibiotics: bacteriocins, antimicrobial peptides and bacteriophages. **Poultry Science**, 82, 640–647.

21. Bruelink, E., van Kraaij, C., Demel, R.A., Siezen, R.J., Kuipers, O.P. & de Kruijff, B. (1997). The C-terminal region of nisin is responsible for the initial interaction of nisin with the target membrane. **Biochemistry**, 36, 6968–6976.

22. Morris, S.L., Walsh, R.C. & Hansen, J.N. (1984). Identification and characterization of some bacterial membrane sulfhydryl groups which are targets of bacteriostatic and antibiotic action. **Journal of Biological Chemistry**, 259, 13590–13594.

23. Abler, L.A., Klapes, N.A., Sheldon, B.W. & Kaenhammer, T.R. (1995). Inactivation of food-borne pathogens with magainin peptides. **Journal of Food Protection**, 58, 381−388.

24. Pihlanto-Leppälä, A. (2001). Bioactive peptides derived from bovine whey proteins: opioid and aceinhibtory peptides. **Trends in Food Science & Technology**, 11, 347−356.

25. Nakai, S., Chan, J. C. K., Li-Chan, E. C. Y., Dou, J. & Ogawa, M. (2003). Homology similarity analysis of sequence of lactoferricin and its derivatives. **Journal of Agricultural and Food Chemistry**, 51, 1215−1223.

26. Ashie, L.N.A., Smith, J.P. & Simpson, B.K. (1996). Spoilage and shell-life extension of fresh fish and shellfish. **Critical Reviews in Food Science and Nutrition**, 36, 87−121.

27. Juven, B.J. & Pierson, M.D. (1996). Antibacterial effects of hydrogen peroxide and methods for its detection and quantitation. **Journal of Food Protection**, 59, 1233−1241.

28. Shin, K., Hayasawa, H. & Lönnerdal, B. (2001). Inhibition of *Escherichia coli* respiratory enzymes by the lactoperoxidase-hydrogen peroxide-thiocynate antimicrobial system. **Journal of Applied Microbiology**, 90, 489–493.

29. García-Graells, C., Opstal, I.V., Vanmuysen, S.C.M. & Michiels, C.M. (2003). The lactoperoxidase system increases efficacy of high-pressure inactivation of foodborne bacteria. **International Journal of Food Microbiology**, 81, 211−221.

30. Touch, V., Hayakawa, S., Yamada, S. & Kaneko, S. (2004). Effects of a lactoperoxidase−thiocyanate−hydrogen peroxide system on *Salmonella enteritidis* in animal or vegetable foods. **International Journal of Food Microbiology,** 93, 175-183.

31. Facon, M.J. & Skura, B.J. (1996). Antimicrobial activity of lactoferrin, lysozyme and EDTA against *Salmonella enteritidis*. **International Dairy Journal**, 6**,** 303−313.

32. Heuvelink, A.E., Zwartkruis-Nahuis, J.T.M., Beumer, R.R. & de Boer, E. (1999). Occurrence and survival of verocytotoxin-producing *Escherichia coli* O157 in meats obtained from retain outlets in the Netherlands. **Journal of Food Protection**, 62, 1115–1122.

33. Venkitanarayannan, K. S., Zhao, T. & Doyle, M.P. (1999). Antibacterial effect of lactoferricin B on *Escherichia coli* O157:H7 in ground beef. **Journal of Food Protection**, 62, 747–750.

34. Chantaysakorn, P. & Richter, R.L. (2000). Antimicrobial properties of pepsin-digested lactoferrin added to carrot juice and filtrates of carrot juice. **Journal of Food Protection**, 63, 376–380.

35. Murdock, C. A. & Metthews, K. R. (2002). Antibacterial activity of pepsin-digested lactoferrin on foodborne pathogens in buffer broth systems and ultra-high temperature milk with EDTA. **Journal of Applied Microbiology**, 93, 850–856.

36. Aasen, I.M., Markussen, S., Møretrø, T., Katla, T., Axelsson, L. & Naterstad, K. (2003). Interactions of the bacteriocins sakacin P and nisin with food constituents. **International Journal of Food Microbiology**, 87, 35–43.

37. Hughey, V.L., & Johnson, E.A. (1987). Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food borne disease. **Applied and Environmental Microbiology**, 53, 2165–2170.

38. Jung, D.S., Bodyfelt, F. & Daeschel, M.A. (1992). Influence of fat and emulsifiers on the efficacy of nisin in inhibiting *Listeria monocytogenes* in fluid milk. **Journal of Dairy Science**, 75, 387–393.

39. Gänzle, M.G., Weber, S. & Hammes, W.P. (1999). Effect of ecological factors on the inhibitory spectrum and activity of bacteriocins. **International Journal of Food Microbiology**, 46, 207–217.

40. Boland, J.S., Davidson, P.M., Bruce, B. & Weiss, J. (2004). Cations reduce antimicrobial efficacy of lysozyme-chelator combinations. **Journal of Food Protection**, 67, 285–294.

41. Abee, T., Krockel, L. & Hill, C. Bacteriocins: mode of action and potentials in food preservation and control of food poisoning. **International Journal of Food Microbiology**, 28, 169–185.

42. Ross, N.L., Sporns, P., Stilles, M.E. & McMullen, L.M. (1999). Inactivation of nisin by glutathione in fresh meat. **Journal of Food Science**, 64, 759–762.

43. de Wit, J.N. & van Hooydonk, A.C.M. (1996). Structure, functions and applications of lactoperoxidase in natural antimicrobial systems. **Netherlands Milk and Dairy Journal**, 50, 227–244.

44. Zapico, P., Medina, M., Gaya, P. & Nunez, M. (1998). Synergistic effect of nisin and the lactoperoxidase system on *Listeria monocytogenes* in skim milk. **International Journal of Food Microbiology**, 40, 35–42.

45. Boziaris, I.S. & Adams, M.R. (2000). Transient sensitivity to nisin in cold-shocked gram-negative bacteria. **Letters in Applied Microbiology**, 31, 233–237.

46. Dykes, G.A. & Moorhead, S.M. (2000). Combined antimicrobial effect of nisin and a listeriophage against *Listeria monocytogenes* in broth but not on raw beef. **International Journal of Food Microbiology**, 73, 71–81.

47. Ulvatne, H., Haukland, H. H., Samuelsen, O., Krämer, M. & Vorland, L.H. (2002). Proteases in *Escherichia coli* and *Staphylococcus aureus* confer reduced susceptibility to lactoferricin B. **Journal of Antimicrobial Chemotherapy**, 50, 461–467.

48. Mazzotta, A.S. & Montville, T.J. (1997). Nisin induces changes in membrane fatty acid composition of *Listeria monocytogenes* nisin-resistant strains at 10°C and 30°C. **Journal of Applied Microbiology**, 82, 32–38.

49. Guo, L., Lim, K.B., Poduje, C.M., Daniel, M., Gunn, J.S., Hackett, M. & Miller, S.I. (1998). Lipid A acylation and bacterial resistance against vertebrate ntimicrobial peptides. **Cell**, 95, 189–198.

50. Helander, I.M., von Wright, A. & Mattila-Sandholm, T.M. (1997). Potential of lactic acid bacteria and novel antimicrobials against gram-negative bacteria. **Trends in Food Science & Technology**, 8, 146–150.

51. Cabo, M.L., Pastoriza, L., Sampedro, G., González, M.P. & Murado, M.A. (2001). Joint effect of nisin, CO_2 and EDTA on the survival of *Pseudomonas aeruginosa* and *Enterococcus faecium* in a food model system. **Journal of Food Protection**, 64, 1943–1948.

52. Leistner, L. (2000). Basic aspects of food preservation by hurdle technology. Review. **International Journal of Food Microbiology**, 55, 181–186.

53. Schillinger, U., Becker, B., Vignolo, G. & Holzapfel, W.H. (2001). Efficacy of nisin in combination with protective cultures against *Listeria monocytogenes* Scott A in tofu. **International Journal of Food Microbiology**, 71, 159–168.

54. McLay, J.C., Kennedy, M.J., O'Rourke, A.L., Elliot, R.M. & Simmonds, R.S. (2002). Inhibition of bacterial foodborne pathogens by the lactoperoxidase system

in combination with monolaurin. **International Journal of Food Microbiology**, 73, 1–9.

55. Pol, I.E., Mastwijk, H.C., Slump, R.A., Popa, M.E. & Smid, E.J. (2001). Influence of food matrix on inactivation of *Bacillus cereus* by combinations of nisin, pulsed ectric field treatment and carvacrol. **Journal of Food Protection**, 64, 1012–1018.

56. Lee, D.U., Heinz, V. & Knorr, D. (2003). Effects of combination treatments of nisin and high-intensity ultrasound with high pressure on the microbial inactivation in liquid whole egg. **Innovative Food Science & Emerging Technologies**, 4, 387–393.

57. Ross, A.I.V., Griffiths, M.W., Mittal, G.S. & Deeth, H.C. (2003). Combining nonthermal technologies to control foodborne microorganisms. **International Journal of Food Microbiology**, 89, 125–138.

58. Kamau, D.N., Doores, S. & Pruitt, K.M. (1990). Enhanced thermal destruction of *Listeria monocytogenes* and *Staphylococcus aureus* by the lactoperoxidase system. **Applied & Environmental Microbiology**, 56, 2711–2716.

59. Knight, K.P., Bartlett, F.M., McKellar, R.C. & Harris, L.J. (1999). Nisin reduces the thermal resistance of *Listeria monocytogenes* Scott A in liquid whole egg. **Journal of Food Protection**, 62, 999–1003.

60. Barrett, N.E., Grandison, A.S. & Lewis, M.J. (1999). Contribution of the lactoperoxidase system to the keeping quality of pasteurized milk. **Journal of Dairy Research**, 66, 73–80.

61. Zapico, P., Paz, M., Medina, M. & Nunez, M. (1999). The effects of homogenization of whole milk, skim milk and milk fat on nisin activity against *Listeria inocua*. **International Journal of Food Microbiology**, 46, 151–7.

62. Laridi, R., Khead, E.E., Benech, R.O., Vuillemard, J.C., Lacroix, C. & Fliss, I. (2003). Liposome encapsulated nisin Z: optimization, stability and release during milk fermentation. **International Dairy Journal**, 13, 325–336.

63. Benech, R.O., Kheadr, E.E., Lacroix, C. & Fliss, I. (2002). Antibacterial activities of nisin Z encapsulated in liposomes or produced in situ by mixed culture during cheddar cheese ripening. **Applied and Environmental Microbiology**, 68, 5607–5619.

64. Were, L.M., Bruce, B.D., Davidson, M.P. & Weiss, J. (2003). Size, stability and entrapment efficiency of phospholipid nanocapsules containing polypeptide antimicrobials. **Journal of Agricultural and Food Chemistry**, 51, 8073–8079.

65. Kaszuba, M. & Jones, M.N. (1999). Hydrogen peroxide production from reactive liposomes encapsulating enzymes. **Biochemistry Biophysica Acta**, 1419, 221–228.

66. Cutter, C.N. & Siragusa, G.R. (1997). Growth of *Brochothrix thermosphacta* in ground beef following treatments with nisin in calcium alginate gels. **Food Microbiology**, 14, 425–430.

67. Gill, A.O. & Holley, R.A. (2000). Surface application of lysozyme, nisin and EDTA to inhibit spoilage and pathogenic bacteria on ham and bologna. **Journal of Food Protection**, 63, 1338–1346.

68. Natrajan, N. & Sheldon, B.W. (2000). Inhibition of Salmonella on poultry skin using protein and polysaccharide-based films containing a nisin formulation. **Journal of Food Protection**, 63, 1268–1272.

69. Scannell, A.G.M., Hill, C., Ross, R.P., Marx, S., Hartmeier, W. & Arendt, E.K. (2000). Development of bioactive food packaging materials using immobilized bacteriocins Lacticin 3147 and Nisaplin®. **International Journal of Food Microbiology**, 60, 241–249.

70. Naidu, S.A. (2002). Activated Lactoferrin — a new approach to meat safety. **Food Technology**, 56, 40–45.

71. Fang, T.J. & Tsai, H.C. (2003). Growth patterns of *Escherichia coli* O157:H7 in ground beef treated with nisin, chelators, organic acids and their combinations immobilized in calcium alginate gels. **Food Microbiology**, 20, 243–253.

72. Appendini, P. & Hotchkiss, J.H. (2002). Review of antimicrobial food packaging. **Innovative Food Science & Emerging Technologies**, 3, 113–126.