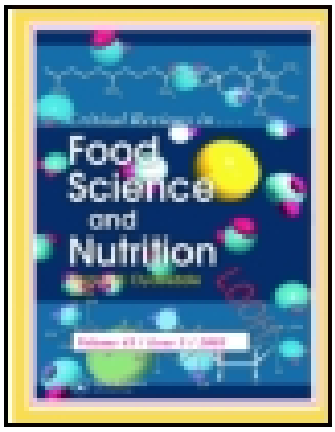

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Nisin as a Food Preservative: Part 1: Physicochemical Properties, Antimicrobial Activity, and Main Uses

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Nisin as a food preservative:

Part 1: Physicochemical properties, antimicrobial activity, and main uses

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Abstract

Nisin is a natural preservative for many food products. This bacteriocin is mainly used in dairy and meat products. Nisin inhibits pathogenic food borne bacteria such as *Listeria monocytogenes* and many other Gram-positive food spoilage microorganisms. Nisin can be used alone or in combination with other preservatives or also with several physical treatments. This article reviews physicochemical and biological properties of nisin, the main factors affecting its antimicrobial effectiveness, and its food applications as an additive directly incorporated into food matrices.

Keywords: Nisin; Bacteriocins; Structure; Action mode; Antimicrobial spectrum; Food preservation.

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1. Introduction

Biological preservation approaches are attractive as a safety parameter in foods with reduced contents of ingredients and additives that usually serve to inhibit microbial growth (Schillinger et al., 1996). Nisin, a small peptide produced by *Lactococcus lactis* ssp. *lactis*, attacks the cell wall and causes lysis of the target microorganisms. Nisin is currently industrially produced and is used for specific applications such as prevention of spore germination and growth of pathogenic bacteria contaminating the surface of food products. This bacteriocin has been commercialized for the first time in the 50ôs to inhibit the outgrowth of *Clostridium tyrobutyricum* responsible for late cheese blowing (García et al., 2010). Lactic acid bacteria (LAB) naturally produce nisin in raw milk and dairy products (Perin et al., 2012), however LAB growth for nisin industrial production requires complex nutrition conditions which increases production costs and complicates the purification steps. This explains the fact that commercial nisin preparations (not exceeding generally 2.5 wt% pure nisin content) are standardized to 10^6 IU g^{-1} with denatured milk proteins and NaCl. If one gram of pure nisin contains 40 10^6 IU, a biological activity of 40 IU corresponds to 1εg of pure nisin whatever the commercial preparation purity. It is noteworthy that the International Unit (IU) corresponds to the amount of nisin able to inhibit one cell of *Streptococcus agalactiae* in 1 mL of broth (Tramer and Fowler, 1964).

Nisin is the only bacteriocin approved as a food preservative, which explains its increasingly common use in food industry. This use is governed by the "FAO / WHO Codex Committee on Milk and Milk Products" which has accepted the use of nisin as a food additive in a concentration not exceeding 12.5 mg of pure nisin per kilogram (Ross et al., 2002). Nisin has

been approved as GRAS (Generally Recognized As Safe) by the US Food and Drug Administration (FDA) since 1988 because it has long been used in food preservation (E234) without being involved in health problems. According to the FDA rules, this bacteriocin can be used at a maximum concentration of 250 ppm in cheese products in order to prevent *Clostridium botulinum* growth (Federal Register, 1988). Nisin is effective against several pathogenic Gram-positive bacteria such as *Listeria monocytogenes* and *C. botulinum*, but also against some Gram-negative pathogens such as *Escherichia coli* and *Salmonella* spp. when combined with chelators such as ethylenediaminetetraacetic acid (EDTA), heat treatment, freezing, or any treatment causing the alteration of the cell wall that becomes permeable which promotes contact between nisin and the cytoplasmic membrane (Belfiore et al., 2007).

The aim of this paper is to discuss available information on nisin structure, physicochemical and biological properties, as well as its applications as a food preservative. Regarding this latter aspect, only the uses of ñfree nisinò will be discussed while nisin incorporation in polymer based materials to produce antimicrobial packaging will be evoked in the 2nd part of this review.

2. Nisin structure

The classification of bacteriocins produced by LAB evolves with the research progress in this field. It is based on several criteria: molecular weight, posttranslational modification, and biological activity. However, serious problems of listeriosis have prompted researchers to consider the antimicrobial activity against *L. monocytogenes* as a classification criterion (Ennahar et al., 2000). The simplest classification includes bacteriocins into three classes: class I (lantibiotics); class II (small, heat-stable and non-lantibiotics peptides); and class III (large and

heat-sensitive proteins). The majority of bacteriocins produced by the bacteria associated with food belong to classes I and II. The most important class of bacteriocins is lantibiotics which contain an unusual amino acid: lanthionine (Lan). Because of this unusual amino acid and its specific biological activity, these peptides are named lantibiotics. More than 25 lantibiotics have been described in literature but the most important and most studied is nisin (Jack and Jung, 2000).

Nisin contains 4 unusual amino acids: dehydroalanine (DHA), dehydrobutyrine (DHB), lanthionine, and ϐ-methyllanthionine (**Figure 1**) that form thioether bridges in five positions. Unlike other proteins, nisin has no absorbance at 280 nm because it does not contain aromatic amino acids. Nisin is a cationic polypeptide, hydrophobic and heat stable. The nisin molecular weight is 3 510 Da but this peptide is capable of forming dimers (7 000 Da, more stable dimer) and tetramers (14 000 Da). Several types of nisin have been identified. The main variants are called A, Z, and Q (**Figure 2**) and possess different biological activities. However, nisin A and Z are the most active forms that are often marketed. These two variants (A and Z) differ by one amino acid at position 27: histidine for nisin A and asparagine for nisin Z. This substitution results in little difference in terms of thermal stability, resistance to pH changes, sensitivity to proteolytic enzymes, and antimicrobial action spectrum (Sonomoto et al., 2000). However, this last study has shown that nisin Z is more soluble than nisin A in pHs near neutrality because asparagine has a more polar side chain than that of histidine. This structural difference does not affect antimicrobial activity but it changes some properties: the nisin Z is more soluble and its distribution in foods has been shown to be higher (Bouksaim et al., 2000).

3. Nisin physicochemical properties

Antimicrobial activity of nisin is largely dependent on its aqueous solubility and structural stability which in turn depend on pH. Indeed, nisin is more soluble and more stable under acidic conditions and has a solubility of 12 wt% at pH 2.5 and 4 wt% at pH 5.0. This solubility is close to zero when the pH reaches and exceeds neutrality (Hurst, 1981). Similarly, the antimicrobial activity is stronger at acidic pH (hydrochloric acid solution pH 2.5 for example) and gradually decreases with increasing pH which can be explained by an irreversible modification of the molecular structure of nisin (Hurst, 1981). This structural modification may remarkably take place at pHs > pI (isoelectric point) (~ 8-9) and occurs primarily through the formation of multimers *via* inter-molecular interactions (Liu and Hansen, 1990).

Nisin activity is highly stable at low temperatures (during freezing for example) but this activity can be lost when the peptide is heated for a long time. In addition, nisin thermostability is largely related to pH. For example, the antibacterial activity of nisin is completely retained at pH 2 after autoclaving at 121 °C but is completely lost after 30 min at 63 °C at pH 11 (Hansen et al., 1991). As a general rule, thermal stability of nisin increased with decreasing pH (Rollema et al., 1995). This can be explained by the presence of the five thioether bridges.

Nisin can also be inactivated by proteolytic enzymes such as pancreatin, Ŭ-chymotrypsin, and ficin that are able to break its peptidic chain. However, other enzymes such as trypsin, pepsin and carboxypeptidase have no significant effect on its antimicrobial effect (Chollet et al., 2008).

These nisin activation/inactivation factors should be seriously considered when nisin is used in food systems that have different values of pH, ionic strength, viscosity, and fat contenté Therefore, the structure, composition, pH, and shelf life of the considered food matrix should be

considered to interpret consistently the resistance of contaminating microorganisms to nisin. For example, food matrices for which the risk of growth of pathogenic bacteria such as *Listeria monocytogenes* is the highest have a pH close to neutrality: one must keep in mind that both solubility and antimicrobial activity of nisin are lower at these pH values than at acidic pH.

4. Nisin biological activity

Many of the nisin sensitive bacteria, such as *Listeria monocytogenes* and *Clostridium botulinum*, are known to be pathogenic. In fact, nisin has antimicrobial activity directed primarily against Gram-positive bacteria and in particular the spore forming ones. For spore-forming bacteria, nisin is able to inhibit both the vegetative forms and the outgrowth of their spores. Other studies also suggest the ability of nisin to inhibit the germination of *Bacillus* and *Clostridium* spores (Hurst, 1981; Venema et al., 1995). Furthermore, nisin can also inhibit some non-spore forming bacteria such as *Staphylococcus*, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Listeria, Pediococcus,* and *Micrococcus*. It should also be noted that nisin has no inhibitory activity against yeast cells, filamentous fungi, viruses and Gram-negative bacteria. In normal circumstances, Gram-negative bacteria are usually resistant to nisin mainly due to their impermeable outer membranes (Gänzle et al., 2003).

The nisin action mechanism is widely studied (McAuliffe et al., 2001) and consists in the adsorption on the target cell surface and destabilization of the cytoplasmic membrane structure. This adsorption involves electrostatic interactions between nisin having a net positive charge and the negatively charged membrane phospholipids (Martin et al., 1996). These electrostatic interactions are also due to the hydrophilic character of the C-terminal extremity of the polypeptide. The hydrophobicity of the N-terminal extremity of nisin acts subsequently to allow

the insertion of nisin in the lipid cell membrane leading to its permeabilization. The effectiveness of this integration depends on the nature and content of the cell membrane phospholipids which may explain differences in sensitivity between target bacterial strains. The release of the essential cytoplasm components, and/or cell lysis, results in the bacterium death (McAuliffe et al., 2001). Association between nisin hydrophobic patches and hydrophobic bacterial membranes was modeled using computer simulation to predict the most favorable interactions for an optimum antimicrobial activity. Probably, it is the hydrophobic part inserted into cell membrane that forms pores (Lins et al., 1999). One of the several modes of nisin action proposed in literature is given in **Figure 3**. Nisin initially forms a complex with Lipid II, a precursor molecule in the synthesis of peptidoglycan forming the bacterial cell walls. This complex then inserts itself into the cytoplasmic membrane forming pores and allows the efflux of essential cellular components resulting in inhibition or death of the bacteria.

5. Nisin applications as food preservative

5.1. Legislation

Several bacteriocins produced by LAB have been purified, characterized and studied for their antibacterial properties. For example, pediocin (Class IIa) possesses a confirmed antilisterial activity (Naghmouchi et al., 2007) and could have several potential applications in food industries. However, nisin is the only bacteriocin which use is currently permitted in food. This peptide was added to the list of food additives under the European number E234 (EEC, 1983). The authorization of the use of this bacteriocin has many reasons: (i) the peptide is easily degradable with intestinal proteases, (ii) it presents no risk to human health, and (iii) does not alter the organoleptic and sensory properties of foods. The legislation concerning the maximum

nisin amount used differs from one country to another: for example, if nisin can be added to the cheese without any limit in the United Kingdom, Australia and France, the amount added should not exceed 100 IU g^{-1} in Belgium, 500 IU g^{-1} in Argentina, and 10 000 IU g^{-1} in the USA (Cleveland et al., 2001). More data concerning the maximum authorized nisin levels are given in **Table 1**. Finally, it is important to highlight that nisin can not be regarded as a "natural" preservative when used in concentrations higher than those naturally found in foods fermented with nisin-producing strains.

5.2. Applications of nisin and related issues

5.2.1. General considerations

Several strategies can be considered for application of nisin for food preservation: (i) inoculation of food with a nisin-producing strain, (ii) use of a fermented product with a nisin-producing strain as an ingredient, (iii) addition of purified or semi-purified nisin as a preservative, and (iv) addition of encapsulated nisin and/or immobilization of nisin in solid matrices (beads, gels, or films) to control its release and protect it from degradation by proteolytic enzymes. Food composition and intrinsic and extrinsic parameters during manufacture, storage, and distribution will determine the appropriate method to be used. In this section we will focus on the direct addition of free nisin to food products.

Nisin can be used in a wide range of liquid or solid foods chilled or stored at room temperature. According to the target microorganisms, areas of nisin use can be classified into three categories: prevention of contamination by lactic acid bacteria, inhibition of pathogenic Gram-positive bacteria such as *Listeria monocytogenes* and prevention of contamination by spore forming Gram-positive bacteria such as *Clostridium botulinum*. Nisin is not active against

other microorganisms and should therefore not be used as a unique preservation agent unless the microflora that is likely to contaminate the food consists mainly of Gram-positive bacteria.

Nisin should preferably be added as an aqueous solution and mixed with food during manufacture. When nisin is added in powder form, one must ensure proper distribution in the food. Alternatively, nisin can be used to decontaminate food surfaces by spraying. During multistage production, nisin must be added during the final step so that it retains its full activity. When making cheese, for example, nisin is added during heating at the same time as melting salts. In practice, nisin is often incorporated into or sprayed on the surface of these products in the form of dry commercial powder containing a small amount (2.5 wt%) of pure nisin.

The used nisin amount depends on many factors: food composition, heat treatment intensity, used packaging, pH, storage time before consumption, and storage conditions. Nisin is often added to acidic foods, but it remains relatively active in a pH range up to 8 (Liu and Hansen, 1990). Nisin is often used for the preservation of pasteurized milk, aged cheeses, and canned soups and vegetables. Nisin can also be used to complement other preservation treatments. In foods stored in cans, nisin is used in addition to heat pasteurization treatments to successfully counter heat resistant spores of flat-sour thermophilic bacteria. When nisin is used, one must be careful to the presence of certain chemical compounds that can alter its biological activity. Indeed, it was shown that titanium dioxide and sodium metabisulphite degrade nisin and inhibit its activity by oxidation of disulfide bridges. Besides, the antibacterial activity of nisin is more potent in a liquid medium than in solid medium (Delves-Broughton, 2005).

The applications of nisin in preventing food microbiological spoilage and pathogenic bacteria proliferation have been widely published and reviewed (Deegan et al., 2006). However,

in the next sections we will summarize some recent common uses of nisin and the related problems and in **Table 2** some examples of these uses.

5.2.2. Meat products

For meat preservation, nitrates are traditionally used: their partial conversion into nitrites by microbial nitrate reductases in fermented meat products allows to prevent *Clostridium* spp. growth. Direct addition of nitrites is an alternative. The reaction of nitrites with secondary amines in meat products can produce significant levels of nitrosamines under certain conditions. Since some nitrosamines are carcinogenic, there is an increasing interest for food preservatives such as nisin, which could replace, even partially, nitrites. However, as mentioned previously in this review, nisin has particular physico-chemical properties influencing especially its stability and biological activity. Thus, when nisin is in contact with meat matrix, it will have an antimicrobial activity that heavily depends on the meat characteristics. The use of nisin in meat is mainly faced to the presence of glutathione, a molecule capable of inactivating nisin by a glutathione S-transferase catalyzed reaction (Rose et al., 1999). This glutathione inactivation is lower in cooked meat due to the loss of free sulphydryl groups, which catalyze the reaction between glutathione and proteins, during heating process (Stergiou et al., 2006). Nisin can also be inactivated by proteolytic enzymes generally found in fresh meat (Rose et al., 1999). In addition to glutathione and the presence of proteolytic enzymes, nisin can easily interact with the meat fats what can reduce its antimicrobial efficacy. In this context, Deegan et al. (2006) explained the fact that nisin is more effective in dairy than in meat products in terms of interactions between nisin and phospholipids. Davies et al. (1999) studied the effect of fat and phospholipids on nisin effectiveness and showed that low fat content was correlated with higher

antimicrobial activity of nisin. Some examples of nisin uses in meat based foods, concentrations, and target microorganisms are summarized in **Table 3**. As a general rule, nisin stability in meat systems during storage depends on four main factors: temperature, pH, presence or absence of intact glutathione and/or active proteases, and storage time.

5.2.3. Dairy products

Nisin addition to milk during cheese making without lactic fermentation allows controlling microbial contamination without nisin alteration during pasteurization. Nisin inhibits the outgrowth of *C. botulinum* spores in cheese spreads (Cleveland et al., 2001). In cheese inoculated with 10^4 CFU (colony forming unit) g^{-1} *Listeria monocytogenes*, a 3 log reduction in *L. monocytogenes* population occurred within 7 days at 20 °C after addition of 50 µg g^{-1} of nisin (Ferreira and Lund, 1996).

The nisin addition at a concentration of 100 mg kg^{-1} completely inhibits spore germination for 3 months during cheese storage at 5 °C. In addition, several species of *Bacillus* have been inhibited by the use of 5 mg kg^{-1} of nisin (Plockova et al., 1996). Study of the shelf life of Ricotta cheese type showed that the use of 2.5 mg L^{-1} of nisin can inhibit the growth of *L. monocytogenes* for more than 8 weeks. In addition, measuring the concentration of residual nisin in cheese showed that the loss does not exceed 32% after incubation at 6-8 °C for 10 weeks (Davies et al., 1997). More examples concerning nisin uses for dairy products preservation are gathered in **Table 4**.

5.2.4. Seafood products

Inactivation of *Listeria innocua* in caviar sturgeon (*Acipenser transmontanus*) and Pacific salmon (*Oncorhynchus keta*) was studied after treatment with nisin and radio frequency heating

or after treatment with antimicrobial chemicals or moderate heating (Al-Holy et al., 2005). Authors found that nisin combined with either radio frequency or moderate heat, inhibited growth of *L. innocua* and *L. monocytogenes* and total mesophilic microorganisms, respectively, without changing the visual quality of treated products. Elotmani and Assobhei (2004) evaluated the inhibition of microbial flora of sardine with nisin and lactoperoxidase, and observed the effectiveness of combining nisin lactoperoxidase in inhibiting the fish spoilage microbiota. More recently, nisin was used to reduce the surface numbers of *L. monocytogenes*, *Salmonella* and native microflora on vacuum-packed raw shrimps stored at 4 °C (Wan Norhana et al., 2012).

5.2.5. Vegetable foods

Cells of *E. coli* and *L. innocua*, used as models for foodborne pathogens, were inoculated into apple or carrot juice (7 log CFU/mL) containing 0 or 10 IU/mL nisin (Pathanibul et al., 2009). In this study, a small amount of nisin (0.25 mg L^{-1}) was added to apple and carrot juices before HHP treatment. No additional inactivation effect was observed in combination with high pressure against *E. coli* K12 cells. However, synergy effects were observed in the case of *L. innocua* under the same conditions. In another study, Xu et al. (2007) suggested that a mixture of nisin with citric acid and grapefruit seed extract could significantly inhibit tested bacteria (three strains of *Salmonella* spp. and three strains of *L. monocytogenes*) and prolong shelf life of fresh-cut ready-to-eat vegetables like cucumber and lettuce. Sun et al. (2012) suggested that nisin can be successfully used for the preservation of fermented vegetable products like beer and wine because it inhibits contaminating bacteria but not influence yeast responsible for the ethanol fermentation. Finally, it is important to note in this section that the major part of the available

literature concerning nisin applications for the preservation of vegetable products uses nisin in association with other chemical conservatives and/or physical treatments.

5.3. Nisin combination with other treatments

5.3.1. Combination with other antimicrobial agents

It has been shown that the effectiveness of nisin increased when combined with other molecules such as lysozyme (Chung and Hancock, 2000), some lactates (Nykänen et al., 2000), essential oils (Razavi Rohani et al., 2011), and listeriophages (Dykes and Moorhead, 2002). In particular, some authors have evaluated the nisin antimicrobial activity in combination with other bacteriocins (Gálvez et al., 2008). A 31 days increase of brined shrimp shelf life was observed after nisin Z in both crude and purified forms addition. Bouttefroy and Millière (2000) tested combinations of nisin and curvaticin 13 produced by *L. curvatus* SB13 to prevent the regrowth of resistant cells of *L. monocytogenes*, believing that this combination induced an inhibitory effect higher than that of a single bacteriocin. Aasen et al. (2003) studied the interactions of sakacin P and nisin with the constituents of cold-smoked salmon, cold-cut chicken, and raw chicken. These authors concluded that due to the amphiphilic nature of these peptides, they can be adsorbed on food macromolecules and undergo proteolytic degradation, which may limit their use as preservatives. Over 80% of added sakacin P and nisin were quickly adsorbed by the food matrix proteins. In non heat treated foods, proteolytic activity caused a rapid degradation of bacteriocins. Less than 1% of the total activity remained after 1 week storage of cold-smoked salmon, and even less in raw chicken. In heat processed foods, bacteriocin activity was stable for over 4 weeks. No significant differences were observed between sakacin P and nisin, but less nisin was adsorbed to muscle proteins at low pH.

Lysozyme is an enzyme commonly added to milk during cheese making with the aim of inhibiting *Bacillus genus* bacteria but has no effect on nisin producers like *Lactococcus* strains. The combination of lysozyme and nisin caused severe cell damage as the authors have observed by scanning electron microscopy compared to samples treated with nisin alone (Chung and Hancock, 2000). These damages reflect the action of lysozyme. In addition, nisin-lysozyme combination caused rapid permeabilization (depolarization) of cytoplasmic membranes of *Staphylococcus aureus*, an effect that reflects the nisin action mechanism. Thus, nisin and lysozyme appear to demonstrate synergy against Gram-positive bacteria because they reinforce the action of each other to kill bacteria. The nisin activity and that of supernatant of a *Bacillus licheniformis* ZJU12 strain showed synergy against 3 food contaminating bacteria: *Staphylococcus aureus*, *Micrococcus flavus*, and *Bacillus cereus* (He and Chen, 2006). However, this study also showed that nisin activity and that of the used supernatant begin within the first 30 minutes while activity of the combination was observed after 4 h.

Lactoperoxidase system (LPS) has a synergistic effect with nisin to inhibit *L. monocytogenes* ATCC 15313 in skim milk and effectiveness of this combination allows to have no detectable cells in 1 mL of milk after 15 days at 25 ° C (Boussouel et al., 2000). The maximum inhibitory effect was observed when nisin was added at the beginning and then LPS was added 4 h later which shows that the addition order is important in the mechanism of action on the cell target membrane. Indeed, nisin forms pores in the membrane after interaction with phospholipids, whereas LPS produces a molecule, hypothiocyanite, which reacts with thiol groups of some proteins important for the viability of pathogenic bacteria, which inactivates the

enzyme systems (Boots and Floris, 2006). The action mechanisms of these two factors can therefore explain the observed synergy.

Combination of nisin (2.5 mg L^{-1}) and monolaurin (250 mg L^{-1}) induces a bactericidal synergistic effect on vegetative cells of 4 tested *Bacillus* species after 5 days at 37 °C (Mansour and Millière, 2001). This bactericidal effect is due to both regrowth and sporulation inhibition. Nisin combination with lactic acid has increased nisin effectiveness to inhibit some Gram-negative bacteria. However, this combination had no effect on inhibition of *L. monocytogenes* (Ariyapitipun et al., 2000). Nykänen et al., (2000) tested the combination of nisin and sodium lactate in the control of *L. monocytogenes* in cold-smoked trout, believing that nisin and sodium lactate injected into smoked fish decreased the number of *L. monocytogenes* cells by 3.3 to 1.8 log CFU g^{-1} after 16 days storage at 8 °C.

The combination of nisin and other bacteriocins with other treatments and other chemicals has also been already well reviewed (Gálvez et al., 2007; Gálvez et al., 2008).

5.3.2. Combination with heat treatments

Nisin addition during heat treatment could be an effective way to increase the shelf life of food products and to use milder treatments (Al-Holy et al., 2012; Li et al., 2012), which help to preserve the organoleptic and sensory properties of these foods. In fact, during sterilization treatments, nisin is known to influence the microorganismôs thermal resistance by changing the value of the D constant (decimal reduction time). In the presence of nisin (25 mg L^{-1}), the average D value of *B. cereus* in milk is reduced by more than 40% in a temperature range of 80-100 °C (Penna and Moraes, 2002). Wandling et al., (1999) showed that the decimal reduction

time of *B. stearothermophilus* ATCC 12980 spores was reduced by 13 and 21% in the presence of 50 mg and 100 mg L^{-1} of nisin at 130 °C, respectively.

Wirjantoro et al. (2001) showed that the addition of nisin (1.875 or 3.75 mg L^{-1}) to milk before sterilization increases the shelf life with or without refrigeration. In this study, no microbial growth was observed in the milks treated at 117 °C for 2 s after storage at 10 or 20 °C for 1 year in addition to their best sensory properties compared to UHT-treated milk. Budu-Amoako et al. (1999) evaluated nisin activity in combination with heat treatment to inhibit *Listeria* in cold-packed lobster meat. They observed 3 to 5 log reductions of inoculated *L. monocytogenes* populations, while heat treatment or nisin alone resulted in reductions from 1 to 3 log.

5.3.3. Combination with other physical treatments

The current trend is the use of nisin in combination with new non-thermal preservation techniques such as high hydrostatic pressure (HHP) (Pathanibul et al., 2009; Zhao et al., 2012) or pulsed electric fields (PEF) treatments (Nguyen and Mittal, 2007). This explains the abundance of literature in this field. HHP treatments can inactivate Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas fluorescens* but Gram-positive ones such as *Listeria innocua* seem to be more resistant (Black et al., 2005). In this latter study, viability of several bacteria was evaluated in skim milk treated at pressures of 250-500 MPa for 5 min at 20 °C in the presence of 0, 6.25, or 12.5 mg $L^{-1}$of nisin. The combination of the two treatments gave higher inactivation than that obtained for each treatment separately for both Gram-negative and Gram-positive bacteria. Nisin addition to milk causes damage to bacteria cell membranes and increases their sensitivity to high pressures.

After treatment of milk containing 12.5 mg L^{-1} at 500 MPa for 5 min, a greater reduction (8 log) was observed for *Listeria innocua* and *Lactobacillus viridescens*. Taken separately, the same physical treatment led to a 3.8 log reduction while the same amount of nisin caused a 1.5 log reduction. Since nisin is effective against Gram-positive bacteria, a sensitization by nisin of Gram-positive bacteria to inactivation by HHP treatments was expected by several researchers. In fact, the microbial reduction of pathogenic bacteria such as *Staphylococcus aureus* or *Listeria monocytogenes* achieved by the combination of HHP treatments and nisin, indicates that, the microbiological safety of food could be improved, while little changing organoleptic properties or less than most of thermal treatments.

Combination of 500 MPa and nisin was the most effective treatment to inactivate indigenous microorganisms of goat cheese (Capellas et al., 2000). The number of aerobic mesophilic microorganisms in the cheese subjected to combined treatment was lower than that obtained in the case of only nisin treated cheese due to the effect of high pressure on cells sensitization. However, this effect was not additional because there are some populations that may be inactivated by both HHP and nisin. Above 150 MPa, *Escherichia coli* became sensitive to nisin when the bacteriocin was added before HHP treatment, but HHP-treated cells remained insensitive to nisin when it was added after treatment. The authors considered that HHP treatment can sensitize *Escherichia coli* to nisin by inducing a transient permeabilization of the outer membrane that does not involve physical disruption and is immediately restored after the process (Diels et al., 2005).

HHP is also an attractive non-thermal process to improve the preservation of meat products. The behavior of several foodborne bacteria in a meat model system containing several

bacteriocins including nisin after pressurization (400 MPa, 10 min, 17 °C) and during cold storage has been studied (Garriga et al., 2002). Among the bacteria studied, *Staphylococcus* was the least sensitive to pressure but in the presence of nisin this bacterium showed a lower cell number during storage at 4 °C than in the presence of other bacteriocins. Greater inactivation of *E. coli* (> 6 log) in the presence of nisin has been registered and the number of surviving cells remained unchanged for 61 days during storage at 4 °C. Masschalck et al. (2000) have succeeded to inactivate the mutant *E. coli* MG1655 strain, known for its high resistance to high hydrostatic pressure, by using a relatively low pressure (400 MPa) in the presence of nisin (2.5 mg L^{-1}). At room temperature, this treatment was able to achieve 6 log reductions. In this work, a hypothetical mechanism of "pressure-promoted uptake" has been proposed to explain the outer membrane permeabilization under pressure by lipophilic cationic peptides like nisin or enzymes like lysozyme.

Gao and Ju (2008) studied the combined effects of pressure (300-700 MPa maintained from 7.5 to 17.5 min at temperatures ranging from 30 to 70 °C) and nisin (0-8 mg L^{-1}) on the inactivation of *C. botulinum* 33A spores. They have pinpointed the optimum process parameters for a 6 log reduction of spores: pressure of 545 MPa; temperature of 51 °C; pressure holding time of 13.3 min; and a nisin concentration of 3.22 μg mL^{-1}.

The effect of combined HHP and nisin treatments on microbial inactivation in liquid whole egg was studied by Lee et al. (2003). The addition of nisin (0.5-20 mg L^{-1}) before pressurization treatments significantly increased the lethal effects of high pressure against *Listeria seeligeri* (up to 5 log-reduction of their population). On the other hand, individual effects of HHP and nisin on *Listeria* were almost negligible, and therefore the observed reductions were

considered to be due to the synergistic action of nisin and HHP. However, study of the effect of the combination of nisin with HHP on *E. coli* showed exactly the same inactivation level by HHP alone, which was interpreted by the authors in terms of protection of Gram-negative bacteria against nisin action.

Lee and Kaletunç (2010) studied effects of nisin (5 mg L^{-1}), HHP (100-150 MPa), and their combination on the cellular components of two pathogenic strains of *Salmonella* Enteritidis (Gram-negative) by differential scanning calorimetry (DSC). They showed that pressure increases the sensitivity of the two strains to nisin. Indeed, high pressure caused alterations in the outer cell membrane thereby facilitating nisin penetration in the cell. The results obtained by DSC were used to compare the different final states of the cells obtained under different treatment conditions starting from the same initial state. The apparent enthalpy of each strain did not change after nisin addition under atmospheric pressure while under high pressure a reduction in enthalpy due to ribosome denaturation was observed.

López-Pedemonte et al., (2003) studied the effect of HHP on the inactivation of *Bacillus cereus* ATCC 9139 spores inoculated in model cheeses made from raw milk, and the effects of nisin addition (0.05 and 1.56 mg L^{-1}). At a pressure of 400 MPa, highest inactivation (~ 2.4 log CFU g^{-1}) was obtained in the presence of nisin (1.56 mg L^{-1}), while lysozyme (22.4 mg L^{-1}) was not able to increase spores sensitivity to high pressure. When studying the sensitivity of spores of *Bacillus subtilis* and *Clostridium sporogenes* PA 3679, nisin showed a synergistic effect with pressurization at high temperatures and acidic pH for both types of studied spores. This effect was most clear at a pressure of 404 MPa, 45 °C, pH 4.0 for 15 min. Under these conditions, the number of *B. subtilis* spores decreased by 3 log and a further reduction of 3.1 log was observed

in the presence of nisin (Stewart et al., 2000). Furthermore, HHP treatment may also improve the efficiency of nisin on the inactivation of some spores by increasing their permeability after the germination process. The number of *Bacillus cereus* spores in a traditional curd cheese was significantly reduced when nisin addition was followed by two HHP cycles, a cycle to induce spore germination and a second to destroy vegetative cells (López-Pedemonte et al., 2003).

Several other studies have been conducted in order to explain the synergy resulting from the combination of HHP and nisin. Among the proposed hypotheses, the pore formation by nisin, followed by its parallel orientation and subsequent binding to the membrane, may increase the susceptibility of microorganisms to pressure by local immobilization of phospholipids (Ter Steeg et al., 1999). Synergistic effects can also be attributed to sub-lethal damage due to cell wall leakage and/or outer membrane, for Gram-negative bacteria, by high pressures which could facilitate the nisin access to the cytoplasmic membrane (Hauben, et al., 1996).

Other physical treatments such as pulsed electric fields were also used in combination with nisin. PEF damage cell walls and membranes that lose their barrier function. Observations by transmission electron microscopy of *L. innocua* cells showed differences in morphology between cells treated with nisin, with PEF, and those treated by the combination of both (Calderón-Miranda et al., 1999). *L. innocua* cells subjected to PEF in skimmed milk containing nisin (0.925 mg L^{-1}) showed an increase in the cell wall width. When applying the highest electric field intensity used in this study (50 kV cm^{-1}), a cell elongation was observed. The combination of PEF and nisin has an additional effect on the morphological damages of *L. innocua* like pore formation. According to Calderón-Miranda et al. (1999), *L. innocua* inactivation is a consequence of the cell membrane breakdown and loss of its functionality. This

synergistic effect is not consistent with other studies that even show that nisin has no additional lethal effect during treatment with PEF. Indeed, Terebiznik et al. (2000) explain that the outer cell wall shrinkage and the cytoplasmic membrane tear facilitate nisin entry in the cytoplasm and loss of its ability to form pores because of the internal pH (alkaline) and chemical potential (negative).

5.3.4. Combination with modified atmosphere

Nilsson et al. (1997) tested the combination of nisin with a CO_2 atmosphere for the control of *L. monocytogenes* in smoked salmon. Results showed that the combination of nisin and CO_2 in the cold-packaged smoked salmon resulted in a reduction of *L. monocytogenes* by 1 to 2 log followed by a lag phase of 8 to 20 days when 500 and 1000 IU nisin/g are used, respectively. However, despite the importance of this non-thermal technique, few studies are published on this subject.

5.4. Factors affecting nisin activity in food systems

When nisin is directly added to foods, its effectiveness can be altered by the physicochemical properties of the system such as high pH, high fat content, and the presence of large particles. These parameters can generate interactions with nisin, precipitation, inactivation, or non-uniform distribution within the food. Nisin effectiveness is thus generally lower in food systems than in growth media. To reach the same efficiency, it is often necessary to add a nisin amount about ten times higher than that in a culture medium (Gálvez et al., 2007). Nisin effectiveness depends also on the food microbial properties like the type of microflora contaminating the food and the properties of the targeted bacteria. The growth phase of microorganisms contaminating the food can influence their sensitivity and therefore the nisin effectiveness. Indeed, cells that are not in a

growth phase may be more resistant. In addition, cells that have already adapted to changes in their environment are often insensitive to bacteriocins. The effectiveness of nisin can also be significantly lower when microorganisms are associated as biofilms (Arevalos-Sánchez, 2012).

EDTA is a chelator of divalent cations (especially Ca^{2+} and Mg^{2+}) that contribute to the stability of the outer membrane of Gram-negative by providing electrostatic interactions between proteins and polysaccharides (Delves-Broughton, 1993). The absence of these interactions exposes hydrophobic phospholipids which increases susceptibility to degradation by antimicrobial agents such as nisin. Nisin, in the presence of EDTA, has a high activity against Gram-negative bacteria such as *Salmonella* (Tu and Mustapha, 2002). The use of some surface active molecules such as Tween 80 helped considerably to keep nisin activity in half-whole milk (Jung et al., 1992). These results were subsequently interpreted by the fact that the amphiphilic Tween 80 is capable of moving nisin from the water/fat interface thereby enhancing its adsorption to cell membranes of bacteria (Bhatti et al., 2004). In addition, it is important to note that nisin can chemically degrade during the production and storage of some food products like cheese (Schneider et al., 2011). In fact, it has been shown that nisin is susceptible to the acid catalyzed addition of a water molecule at the double bond of the unsaturated aminoacids.

Nisin use is also limited by the emergence of resistant strains and its ineffectiveness is not genetically related to the nisin production itself. Nisin-resistance mechanisms are strain-specific. Jarvis and Farr (1971) explained the resistance of *Bacillus cereus* by inactivation of nisin through a reductase that acts on dehydroaminoacids. This resistance can also occur spontaneously in some nisin sensitive mutant strains that are grown in the presence of this lantibiotic. The mechanism of this resistance may reflect the synthesis of a lipoprotein able to "obstruct"

membrane pores formed by nisin (Engelke et al., 1994). This resistance phenomenon, due to genetic mutations, is a major problem that limits the use of nisin as a food preservative. For example, *Streptococcus thermophilus* INIA 463 is a nisin sensitive strain but became resistant after exposure to low nisin concentrations (1-3 IU mL^{-1}) for less than 2 h. This resistance is due to an increase in the thickness of the cell wall as revealed by transmission electron microscopy (TEM). Results also showed that resistance disappears after 4 h of growth in skim milk (Garde et al., 2004). Some strains of *L. monocytogenes* showed resistance to nisin in acidic conditions and a study has also demonstrated that this resistance does not depend only on pH but also the nature of the used acid (Bonnet and Montville, 2005).

6. Conclusions and future research needs

Nisin use for food preservation may offer several advantages: (i) increasing the shelf life of the product, (ii) reducing the transmission risk of pathogen food borne, (iii) reducing the use of salts, acids, and other chemical preservatives, and (iv) permitting the use of soft treatments which better preserve vitamins and organoleptic properties. However, the majority of articles concerning nisin applications deals with its combination with other treatments. Because of the rapid reduction of nisin inhibitory activity due to its degradation by proteolytic enzymes or its interaction with fat compounds, current research works aim at improving nisin stability and efficiency by its incorporation in microcapsules, liposomes, or packaging films.

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Table 1. Examples of world-wide maximum level use of nisin

Country	Food in which nisin is permitted	Maximum level (IU g^{-1})
Argentina	Processed cheese	500
Australia	Cheese, processed cheese, canned tomatoes	No limit
Belgium	Cheese	100
EU	E234, labeled as ñnatural preservativeò	may also varies according to product state and member
France	Processed cheese	No limit
Italy	Cheese	500
Mexico	Nisin is a permitted additive	500
Netherlands	Factory cheese, processed cheese, cheese powder	800
Russia	Dietetic processed cheese, canned vegetables	8 000
UK	Cheese, canned foods, clotted cream	No limit
US	Pasteurized processed cheese spreads	10 000

Source: Adapted from Cleveland, Montville, Nes, & Chikindas (2001).

Table 2. Some nisin uses in food applications

Food	Target bacteria	Effective nisin concentration (IU mL^{-1} or IU g^{-1})	References
Cottage cheese	*L. monocytogenes*	2 000	Ferreira & Lund (1996)
Ricotta cheese	*L. monocytogenes*	100	Davies *et al.* (1997)
Skim milk	*B. cereus spores*	4 000	Wandling *et al.* (1999)
Bologna-type sausage	*Lb. sake and Lb. curvatus*	1 000	Davies *et al.* (1999)
Processed cheese	*Clostridium* spp.*;* *Bacillus* spp.	200 ï 600	Delves-Broughton (2005)
Milk products	*Clostridium* spp.; *Bacillus* spp.	10 ï 400	Delves-Broughton (2005)
Pasteurized soups	*B. cereus*	100 ï 250	Delves-Broughton (2005)
Crumpets	*B. cereus*	160 ï 250	Delves-Broughton (2005)
Canned foods	*C. botulinum*	100 ï 200	Delves-Broughton (2005)
Dipping sauces	LAB	50 ï 250	Delves-Broughton (2005)
Beer	*Lactobacillus*, *Pediococcus*	1 000 ï 1 500	Delves-Broughton (2005)

Table 3. Examples of nisin uses for meat products preservation

Food	Nisin concentration	Target microorganisms	Main observations	References
Raw buffalo minced meat	10 or 20 mg kg^{-1}	*Listeria monocytogenes*	*L. monocytogenes* growth inhibition more pronounced at 4 °C than at 37 °C	Pawar *et al.* (2000)
Round steak	Beef meat cubes dipped for 10 min in 125 mg L^{-1} nisin solutions	*Brochotrix thermosphacta*	Once vacuum packed, *B. thermosphacta* growth was inhibited for more than 25 days at 4 °C	Tu & Mustapha (2002)
Cooked sausage	11.25 mg kg^{-1}	No antimicrobial activity assay	More than 68% of initially added nisin still detected after 28 days storage at 6 °C	Reunanen & Saris (2004)
Sous vide cooked (11 min, 97°C) seasoned beef	2.5 or 12.5 mg kg^{-1}	*Bacillus cereus, Clostridium perfringens*	Increase of shelf life at 4°C and/or increased stability in case of temperature abuse (simulated by a storage at 25 °C)	Paik *et al.* (2006)
Meat emulsions (fresh lean beef, 20% w/w bovine fat, 2.5% w/w NaCl)	0.25 to 2.5 mg kg^{-1}	*Listeria monocytogenes*	Time to increase initial *L. monocytenes* population from 10^4 UFC.g^{-1} to 10^7 UFC.g^{-1} at 20 °C increased from 1 to 7 days	Pellicer *et al.* (2011)
Natural sausage casings	Dipping for 8 days in 50 mg L^{-1} nisin solutions	*Clostridium sporogenes*	90% reduction of *C. sporogenes* spores compared with a control	Wijnker *et al.* (2011)

Table 4. Examples of nisin uses for dairy products preservation

Food	Nisin concentration	Target microorganisms	Main observations	References
UHT skim milk	≤ 2.5 mg L^{-1}	*L. monocytogenes*	After an initial effectiveness, a regrowth of nisin-resistant cells was observed.	Zapico *et al.* (1998)
Fresh, pasteurized and/or homogenized whole and skim milk	≤ 12.5 mg L^{-1}	*Listeria innocua* *Listeria monocytogenes*	Antilisterial activity lost or reduced when whole milk was homogenized	Bhatti *et al.* (2004)
Traditional Greek whey cheese	≤ 12.5 mg kg^{-1}	*Listeria monocytogenes*	Direct nisin addition to whey allowed to prevent the increase of *L. monocytogenes* population for more than 30 days when cheeses were stored at 4 °C under vacuum. However, addition of nisin reversed the dominant flora from Gram-positive (lactic acid bacteria) to Gram-negative bacteria along refrigerated storage.	Samelis *et al.* (2003)
Skim milk	≤ 0.5 mg L^{-1}	*Streptococcus thermophilus*	Exposure to subminimal inhibitory concentrations of nisin induced resistance to nisin (possibly caused by changes in *S. thermophilus* cell wall)	Garde *et al.* (2004)
Processed cheese	2.5-12.5 mg kg^{-1}	*Clostridium*	Nisin was effective in delaying or preventing	Delves-Broughton

products		*sporogenes, C. butyricum, C. tyrobutyricum* or *C. botulinum*	growth and subsequent toxin production by inoculated spores of *C. botulinum* types A and B	(2005)
Eweôs milk Greek soft acid-curd cheese	3.75 mg kg^{-1}	lactobacilli, lactococci and yeasts	- Nisin addition induced a significant and expected decrease of lactococci, lactobacilli and an unexpected decrease of yeasts. - Nisin did not affect sensory quality and extended shelf life based on this criterion.	Kykkidoua *et al.* (2007)
Swiss-type (Emmental) cheese	1 g L^{-1} in cheese-water (2:1, w:w) slurry	*Kocuria rhizophila*	Nisin antimicrobial activity reduction can be ascribed to its adsorption on fat globules and proteins but not to its hydrolysis by proteolytic enzymes.	Chollet *et al.* (2008)
Reconstituted powdered infant milk formula	≤ 40 mg L^{-1}	*Cronobacter* spp.	Nisin was not effective likely because they are Gram-negative bacteria and because of the presence of fat globules and proteins	Al Nabulsi *et al.* (2009)
Minas Serro cheese	≤ 12.5 mg L^{-1} (nisin addition to milk before enzymatic coagulation)	*Staphylococcus aureus*	- Up to 2 log cycles reduction in *S. aureus* count from the 7^{th} day of ripening - Decrease of the ripening index	Soares Pinto *et al.* (2011)

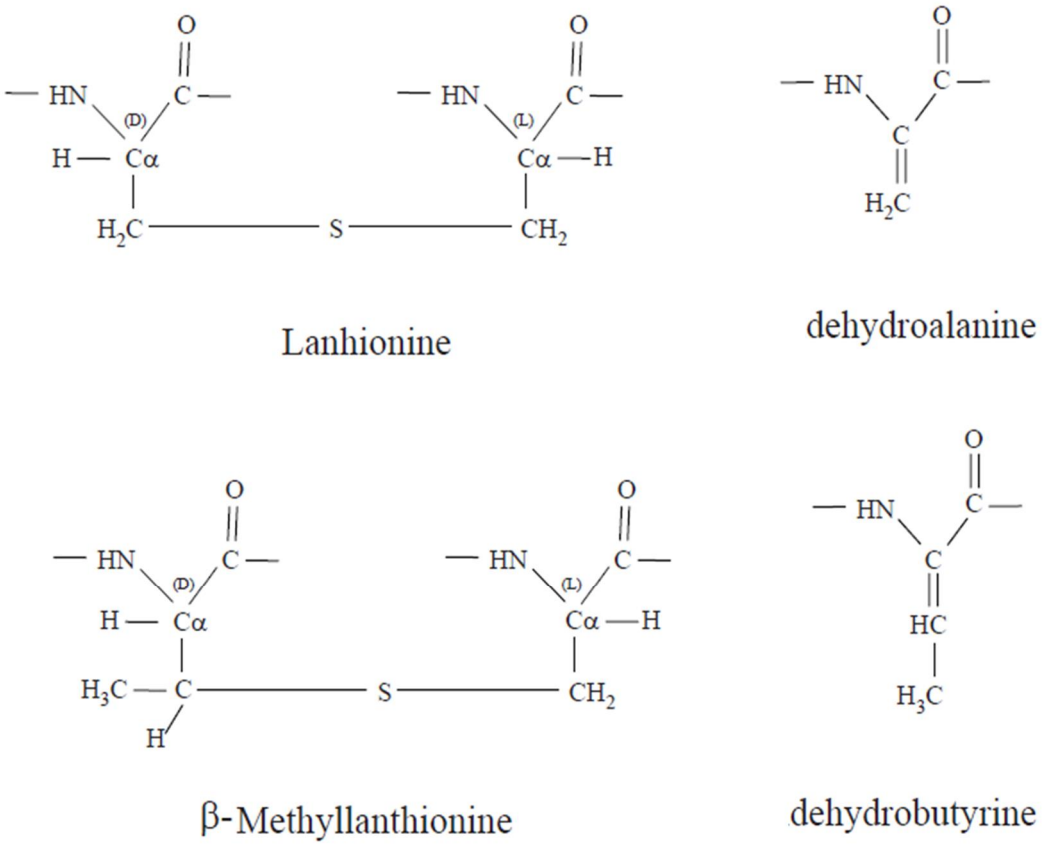

Figure 1. Amino acids characteristic for lantibiotics.

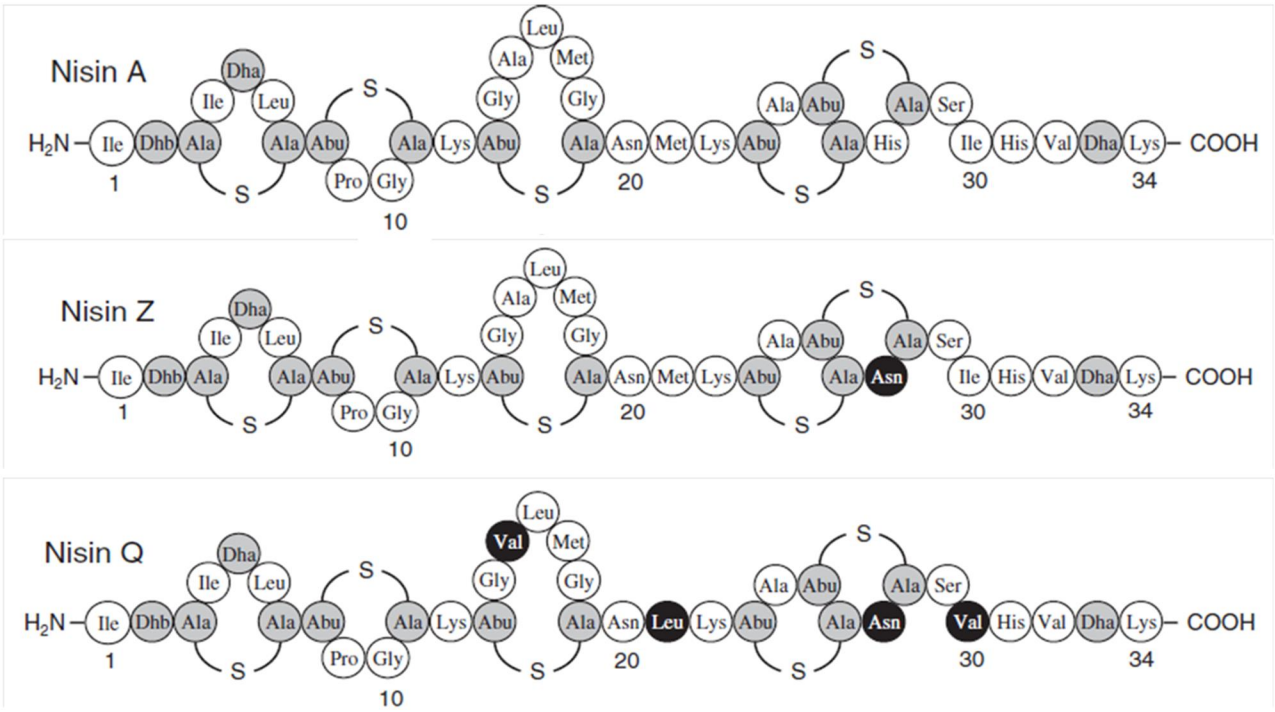

Figure 2. Main natural nisin variants. The black-filled residues indicate the substituted residues as compared with nisin A. The grey-filled residues indicate unusual amino acids.

Ala-S-Ala, lanthionine; Abu-S-Ala, 3-methyllanthionine; S, the sulfur atom of the thioether linkage. (Adapted from: Fukao *et al.*, 2008).

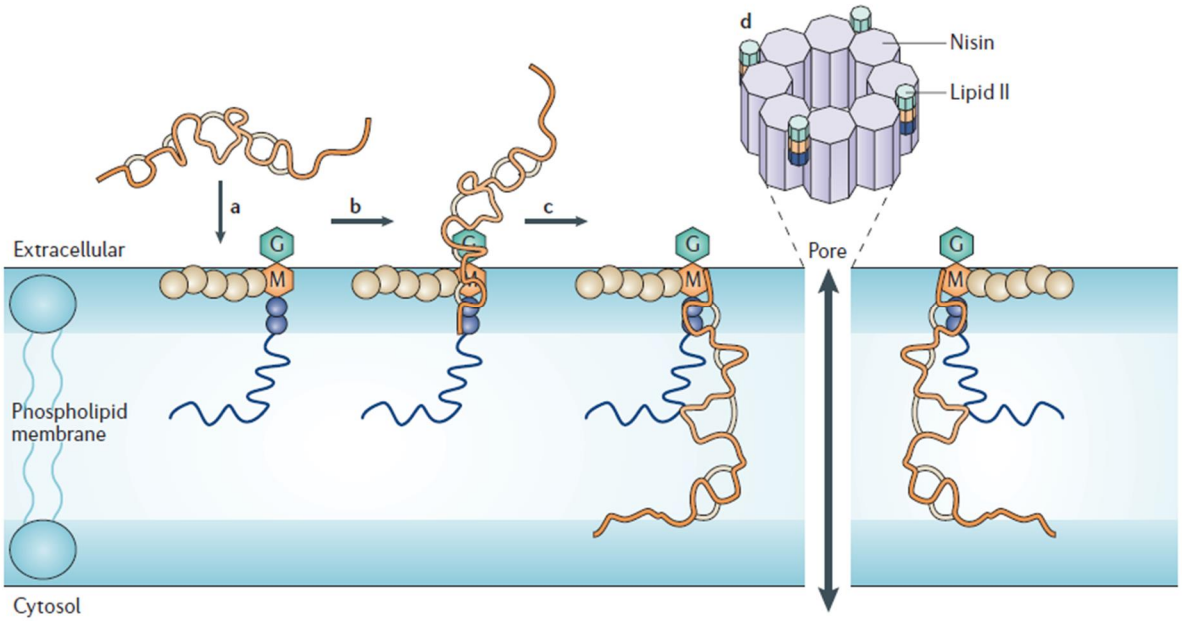

Figure 3. A schematic representation of the mechanism of action of nisin (from Breukink & de Kruijff, 2006). First, nisin reaches the bacterial plasma membrane (**a**), where it binds to Lipid II via two of its amino-terminal rings (**b**). This is then followed by pore formation (**c**), which involves a stable transmembrane orientation of nisin. During or after assembly of four 1:1 (nisin: Lipid II) complexes, four additional nisin molecules are recruited to form the pore complex (**d**).