AN ABSTRACT OF THE THESIS OF

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Title : Application, Interaction, and Enhancement of the Efficacy of Nisin in Foods and Beverages

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Nisin, an antimicrobial peptide, was evaluated in its ability to control bacterial spoilage and malolactic fermentation in wines. Nisin (100 IU/ml) prevented malolactic fermentation in wines. Furthermore, nisin-resistant mutants of *Leuconostoc oenos* were obtained and used to conduct a pure culture malolactic fermentation in the presence of nisin in wines. Nisin's potential to control malolactic fermentation and to inhibit spoilage bacteria was stable in white wines for several months, but decreased significantly within 4 months in red wines.

The interaction of nisin with food components, especially with lipids in milk, was investigated. The activity of nisin against *Listeria monocytogenes* in fluid milk was directly dependent upon the fat content with increasing concentrations decreasing the efficacy of nisin. However, isolated milk fat from half-and-half cream did not interfere with nisin activity when reconstituted into skim milk. Major triacylglycerols of milk fat did not exhibit an antagonistic

effect against nisin activity, but phospholipids interfered with nisin activity. When phospholipids were combined with triacylglycerols, they had a much greater antagonistic effect. Fatty acids also interfered with nisin activity with methylated fatty acids being less influential than unmethylated fatty acids.

Interaction of nisin with certain food components was found to diminish the efficacy of nisin as a food preservative in food systems. To enhance or restore the reduced nisin activity in certain foods, emulsifiers and metal ions were introduced with nisin in foods and beverages. The emulsifier, Tween 80, and calcium ions partially counteracted the decrease of nisin activity in milks. Calcium ions also restored nisin activity in red wine and certain high fat containing foods. Sodium ions did not give a restorative effect to nisin. The enhancement effect of magnesium ions on nisin activity in food systems was between that of sodium and calcium ions. However, nisin was inactive against *Pediococcus pentosaceus* in MRS broth in the presence of divalent cations such as Ca^{2+} or Mg^{2+}. These results suggest that exogenous divalent cations may compete with positively charged nisin molecules for binding to the phospholipids and/or anionic components of foods which interfere with nisin activity, or to the bacterial membrane phospholipids which is believed to be the primary target for nisin action.

APPLICATION, INTERACTION, AND ENHANCEMENT OF THE EFFICACY OF NISIN IN FOODS AND BEVERAGES

by

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INTRODUCTION

Food can be spoiled or its safety compromised due to the growth or presence of pathogenic and spoilage organisms and/or to the presence of toxins produced by microorganisms. Modern society has stimulated development of a variety of processed foods such as frozen and refrigerated foods. Accordingly, the use of chemical preservatives has increased to ensure the safety and shelf life of processed foods. However, today's consumers desire minimally processed foods with freshness and high quality. Furthermore, the public concern about the safety of chemical preservatives and additives has increased and consumers prefer foods that have no preservatives or contain natural preservatives. Therefore the potential use of antimicrobial peptides (bacteriocins), produced by food grade bacteria, as natural food preservatives is becoming attractive to the food industry. Lactic acid bacteria bacteriocins such as nisin and pediocin, exist in certain fermented foods and have been consumed unintentionally for a long time.

Nisin has shown the most promise as an effective bacteriocin food preservative, however its application is limited to certain foods such as dairy products and canned foods. Since nisin is a biologically and chemically active compound, its properties can be altered in a food system due to the interaction of

nisin with food components or surrounding systems. However, limited work has been done to determine the reactivity and stability of nisin in various food systems.

Therefore, the major objectives of this study were :

1) to determine the suitability of nisin's application in wines to control bacterial spoilage and malolactic fermentation.

2) to determine the interactions of nisin with some food components, and their effect on the efficacy of nisin in foods and beverages.

3) to measure the enhancement of the efficacy of nisin by some metal cations in certain food systems.

4) to investigate how data from the above objectives contribute to the current models that address the mode of action of nisin.

LITERATURE REVIEW

NISIN

Nisin is a member of potent antimicrobial substances called bacteriocins. Bacteriocins are proteins or protein containing macromolecules produced by bacteria and exert a bacteriocidal mode of action against susceptible bacteria (Tagg, et al.,1976).

Nisin was discovered by Rogers and Whittier (1928). They demonstrated that lactic acid bacterium *Streptococcus lactis* produced an inhibitory substance which inhibited the growth of other *Streptococci* and of *Lactobacillus bulgaricus*, diffused through a collodion membrane and was inactivated by trypsin. Whitehead (1933) and Cox and Whitehead (1936) also isolated this same inhibitory substance, while investigating the production of "slow-acid milk".

Shattock and Mattick (1943) identified the strains producing this substance as *Streptococcus lactis* (now, *Lactococcus lactis*) strain belonging to Lancefield Group N and the latter workers gave it the name of nisin, derived from Group N Inhibitory Substance, followed by the usual suffix "in" for an antibiotic (Mattick and Hirsch, 1947).

Nisin's application in food preservation was first suggested by Hirsch et al. (1951) who showed nisin could prevent clostridial gas formation in Swiss type cheese, and subsequently nisin was found to be most effective in preventing

clostridial spoilage in pasteurized processed cheese (McClintock et al.,1952). These promising results have stimulated studies for its possible applications in various foods, and indicated that nisin could probably be used as a food biopreservative to control the growth of Gram positive spoilage bacteria. Since 1960, various aspects of nisin have been studied, covering both basic and applied areas. Some of the important studies include elucidation of the amino acid sequence of this 34-amino acid peptide by Gross and Morell in 1971, determination of conformation of the molecule (Barber et al.,1988; Palmer et al.,1989; Van de Ven et al.,1991), cloning and sequencing of the gene encoding for nisin production phenotype (Buchman et al.,1988; Kaletta and Entian,1989; Dodd, et al.,1990; Steen et al.,1991) and testing of its non-toxicity to animals (Hara et al.,1962; Frazer et al.,1962). The results of the applied studies have encouraged its commercial use in several food systems in more than 40 countries and its production and marketing by commercial manufacturers.

CHEMICAL AND PHYSICAL PROPERTIES OF NISIN

Structure of Nisin

The complete structure of the nisin molecule (Fig. 1) was elucidated by Gross and Morell (1971). Nisin consists of 34 amino acid residues and a molecular weight of 3500 Daltons. Nisin can occur as dimers and tetramers with

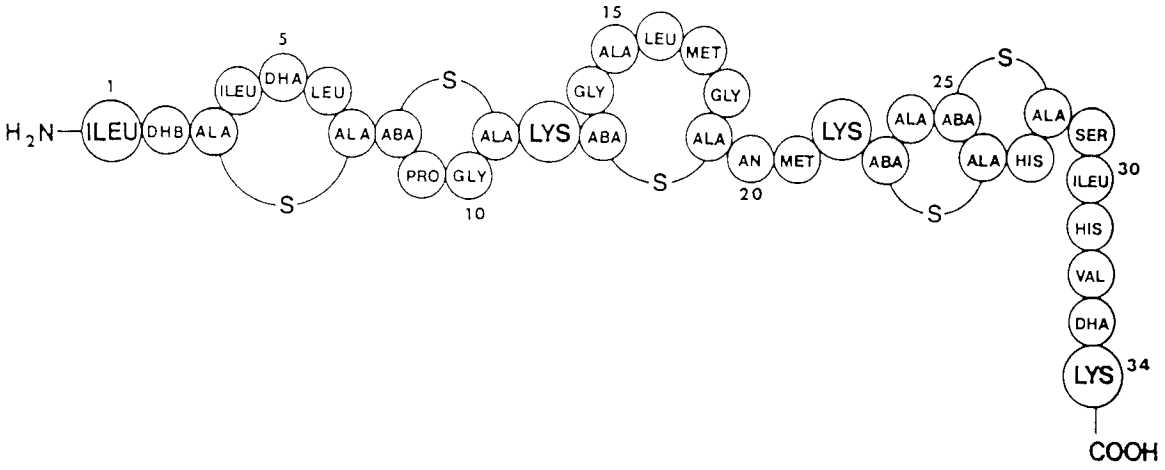

Fig. 1. The structure of nisin, ABA = aminobutyric acid; DHA = dehydroalanine; DHB = dehydrobutyrine (ß-methyldehydroalanine); Ala-S-Ala = lanthionine; Ala-S-ABA = ß-methyllanthionine. from Gross and Morell (1971)

a molecular weight of 7000 and 14,000, respectively. All three forms exhibit biological activity (Jarvis et al.,1968).

Nisin belongs to a class of antimicrobial polypeptides which are known as lantibiotics (Schnell et al.,1988). These polypeptides are characterized by their cationic properties and the high content of unusual amino acids which are posttranslational processing products of ribosomally synthesized precursor polypeptides (Buchman et al.,1988; Schnell et al.,1988). Nisin, the most well studied lantibiotic, contains a high content of unusual amino acids, such as lanthionine, ß-methyllanthionines, dehydroalanines (DHA, residues 5 and 33) and dehydrobutyrine (DHB, residue 2). These atypical residues are derived from serine, threonine and cysteine residues in the precursor peptide via a series of posttranslational enzymatic modifications (Dodd et al.,1990). The modifications involve dehydration of the serines and threonines to produce ∂,ß-unsaturated amino acids, followed by the reaction of some of these residues in a stereoselective way with the sulfhydryl group of the cysteines to create the lanthionines (Kusters et al.,1984; Weil et al.,1990). Nisin contains five rings, each closed by thiolether-bridged lanthionines. The ∂- carbon atoms of the N-terminal units of the five lanthionines are always in the D configuration (Gross and Morell,1971) and ß-carbon atoms of the ß-methyllanthionines are in the S configuration (Kusters et al.,1984; Fukase et al.,1988).

Mulders et al. (1991) recently reported that *L. lactis subsp. lactis* NIZO 22186 produces a form of nisin (nisin Z) which differs from nisin in one amino

acid; the amino acid in position 27 is Asn in nisin Z, but the component is His in nisin.

Nisin has now been produced synthetically by Fukase et al.(1988). Chan et al.(1989) have isolated and characterized two degradation products from nisin, one of which, nisin$^{1-32}$, is active and the other, [des-Δ-Ala-5] Nisin$^{1-32}$, shows little or no activity. This finding suggested that ΔAla33 (or DHA 33) and Lys 34 at the C-terminal were not required for antimicrobial activity of nisin, but ΔAla5 (or DHA 5) was important for the antibacterial properties. Earlier studies (Berridge et al.,1952; Gross and Morell,1967; Gross and Morell,1968) showed that the reduced activity or loss of activity, could be due to the formation of [des ΔAla 5] Nisin$^{1-32}$. Gross and Morell (1970; 1971) suggested that the biological activity of nisin could be due to the DHA (or ΔAla) residues, as they could react with compounds that were metabolically important, such as sulfhydryl-containing enzymes, and thus produce an antimicrobial effect. In addition, ∂,ß-unsaturated amino acids can invoke ion-binding capacity by the polycyclic structure of nisin and play an important role in its biological functions. Recent studies by Liu and Hansen (1990) have also supported the idea that dehydro-residues can play an important role on the antimicrobial property of nisin by reacting with one or more nucleophiles in a sensitive cellular target. It inactivates sulfhydryl residues in germinated bacterial spores (Morris et al.,1984). After reacting nisin with nucleophilic compounds to modify the dehydro groups in DHA and DHB residues, the antimicrobial property of the

molecules is lost. Dehydro residues of nisin, by acting as electrophilic acceptors towards nucleophiles in targets of sensitive cells, are believed to potentiate antibacterial action.

Solubility and Stability

Nisin is insoluble in nonpolar solvents. It is soluble in aqueous media, and the solubility and stability of nisin depend on the pH of the solution. The nisin molecule is a closed polypeptide ring with cationic properties. It is suggested that with increasing pH nisin molecules tend to polymerize, or infolding of single molecules occurs. Polymerizing or infolding of nisin molecules cause blocking of active groups. So the reduction in activity occurs as the pH is raised. At pH 2.5, the solubility of nisin is 12%, and decreases to 4% at pH 5.0, and it is practically insoluble at neutral and alkaline pH values. In a technical information sheet from Alpin and Barrett Ltd.(Anon.,1988), it was reported that the solubility of nisin is 118 mg/ml in 0.02 N HCl (4.7×10^6 IU/ml), 50 mg/ml in distilled water (2×10^6 IU/ml), and 87.5 mg/ml in nonfat milk (3.5×10^6 IU/ml).

The stability of nisin is related to its solubility. In diluted HCl solutions at pH 2.5 or less, nisin can be boiled without loss of activity, and autoclaving of nisin does not cause "serious loss of activity" (Hall,1966). Above pH 7.0, irreversible inactivation occurs even at room temperature. Hall (1966) stated that nisin has a higher antibiotic activity when dissolved in 0.02 M HCl than when assayed for solutions in distilled water. It may be that the tendency to polymerize

is increased by increasing pH, but the relationship between biological activity and polymeric forms of nisin has not been established.

Tramer (1964) reported that nisin was stable at autoclaving at 115.6 °C at pH 2.0, but 40% of the activity was lost at pH 5.0 and more than 90% was lost at pH 6.8. Large macro molecules in milk or broth had a protective effect so that the degree of inactivation is less drastic in foods than in buffer.

The effect of processing temperature and pH on the stability of nisin in different foods was studied by Heinemann et al.(1965). Their results indicated that in low acid foods at pH 6.1 - 6.9, heating for 3 min. at 250 °F destroyed 25-50% of the added nisin. A similar degree of destruction was reported for highly acid foods having pH values of 3.3 - 4.5.

Purified nisin stored as a dry powder in the refrigerator keeps its activity indefinitely, but nisin activity is gradually lost in foods (Hurst,1983). It has been observed in processed cheese (McClintock et al., 1952), canned mushrooms (Denny et al., 1961), chocolate milk (Fowler and McCann, 1971) and simulated cooked ham (Rayman et al.,1981). Losses are more pronounced at high pH and high temperatures.The information on nisin stability indicates that reduction of nisin activity is to be expected depending on heat processing, pH, substrate, and likely temperature and length of shelf life, and that such losses must be compensated for the original addition level.

Nisin is not inactivated by all the proteolytic enzymes. It was found that only ∂-chymotrypsin inactivates nisin, and that the activity exhibited by

pancreatin and trypsin is due to traces of ∂-chymotrypsin (Lipinska, 1977). According to Gross (1975), ∂-chymotrypsin breaks the peptide bond between residues 31 and 32 (see Fig.1). It has been claimed that nisin is inactivated by ptyalin of the saliva. The absence of nisin in the human oral cavity 10 minutes after ingestion of milk containing 200 IU/ml may also support this observation (Claypool et al.,1966; Cowell et al.,1971). Trypsin, elastase, carboxypeptidase A, pepsin and erepsin and similarly proteases of *Aspergillus oryzae* and *Aspergillus parasiticus* , do not inactivate the antimicrobial activity of nisin (Lipinska, 1977).

EFFECTIVENESS OF NISIN IN CONTROLLING PATHOGENS AND SPOILAGE BACTERIA

Units of Nisin Measurement

The activity of nisin is expressed in terms of international units (IU). Originally, Tramer and Fowler (1964) defined a Reading Unit (RU) as the amount of activity exhibited by 1 μg of a standard batch of commercial nisin. This standard was accepted by the World Health Organization (WHO), so that Reading Units are now referred to as International Units (IU) with conversion of 1 RU = 40 IU. However, several methods are also used to report the level of nisin such as IU/ml, μg/ml, ppm, or % nisin in test systems.

Antimicrobial Properties of Nisin

The spectra of activity of nisin is limited to Gram-positive bacteria. It does not inhibit Gram-negative bacteria, yeasts or fungi. Nonetheless, The Gram-positive group of bacteria includes many serious food-borne pathogens and spoilage organisms. Thus within the Gram-positive bacteria, nisin inhibits certain species of *Staphylococcus* (Gowans et al., 1952), *Streptococcus*, *Micrococcus* and *Lactobacillus* (Ogden and Tubb, 1985; Radler, 1990) and the majority of spore forming species of *Clostridium* and *Bacillus* with the spores being more sensitive than the vegetative cells (Mattick and Hirsch, 1947; Campbell and O'Brien,1959; O'Brien et al.,1956). Recent studies (Benkerroum and Sandine,1988; Harris et al.,1991) have shown that strains of *Listeria monocytogenes* , a foodborne pathogen, are sensitive to nisin. Recently, several observations showed that nisin can be effective against Gram-negative bacteria under certain conditions.

Gram-positive Bacteria and Spores

Nisin has been observed to be variable in its inhibitory ability against spores of different bacterial species. Jarvis (1967) demonstrated that spores of certain strains of *Bacillus stearothermophillus* are sensitive to less than 2 IU/ml and that the outgrowth of spores of other *Bacillus* species is inhibited by 3-13 IU/ml of nisin. Some *Bacilli* seem to be more resistance to nisin with *B. cereus* requiring 75-100 IU/ml, *B. megaterium* requiring 25-100 IU/ml (Gupta et al.,

1972; Jarvis, 1967) and *B. polymyxa* requiring 50 IU/ml (Jarvis, 1967) to inhibit spore outgrowth.

Practical attention has been paid to the sensitivity of *C. botulinum* spores to nisin. Sensitivity of *C. tyrobutyricum* to nisin was first addressed by Hirsch in 1951. A number of studies showed the anticlostridial activity of nisin and Hurst (1972) cited *C. botulinum* as one of the more nisin resistant species among species of the *Clostridia* . Scott and Taylor (1981) demonstrated that nisin is effective in preventing the outgrowth of *Cl. botulinum* Types A, B and E spores in bacterial media. Type E spores were the most sensitive with the order of sensitivity to nisin being Type E greater than Type B greater than Type A. The inhibitory levels of nisin were 500 to 2,500 IU/ml for the Type A and B spores and 50 to 1,000 IU/ml for the Type E spores.

Nisin has been reported to inhibit *Listeria monocytogenes*, a notorious food-borne pathogen. However, *Listeria* species exhibite various sensitivity to the level of nisin, depending on the particular strains. Benkerroum and Sandine (1988) reported that three strains of *Listeria monocytogenes* were variably sensitive to nisin and showed different MIC values ranging from 740 to 105 IU/ml in trypticase soy agar and from 1.85 to 103 IU/ml in MRS agar, depending upon the strains tested. Monticello and O'Connor (1990) observed that viable cell numbers of *L. monocytogenes* decreased from about 10^6/ml to about 10^3/ml within 2-3 hrs with 100 IU/ml of nisin in buffer systems. Jung et

al. (1992) also demonstrated that the growth of *L. monocytogenes* Scott A and Jalisco was inhibited by nisin in BHI broth and in skim milk.

Gram-negative Bacteria

In 1947, Mattick and Hirsch concluded from their studies that the Gram-positive bacteria were sensitive to nisin and that it did not inhibit growth of yeasts, molds and Gram-negative bacteria, except for a few strains of *Neisseria.* Linnett and Strominger in 1973 reported that, in a cell free system, 1600 IU/ml of nisin caused 50% inhibition of peptidoglycan synthesis of *Escherichia coli*, and *B. stearothermophilus* . Ogden et al. (1988) observed that three *Flavobacterium* strains and one *Hafnia* strain isolated as brewing contaminants were slightly sensitive to nisin. In 1986, Kordel and Sahl reported that *E. coli* became sensitive to nisin when the outer membrane of cells was disrupted. Blackburn et al. (1989) showed that nisin in 0.1 to 300 μg/ml along with 0.1 to 20 mM EDTA prevented growth of Gram-negative bacteria including *Salmonella spp., Escherichia coli, Pseudomonas spp., Bacterioides spp.,* and *Klebsiella spp* .. Sensitivity of *Salmonella* and other Enteric pathogens to nisin following EDTA treatment has also been recently reported (Stevens et al.,1991, 1992). Ray (1992) also reported that nisin sensitivity of Gram-negative bacteria, including *Salmonella spp., Yersinia spp., Aeromonas spp., pathogenic Escherichia coli* and *Pseudomonas spp.* following freezing, heating, low pH and EDTA treatment. They explained that the developed sensitivity of Gram-negative

bacterial cells to nisin is a consequence of sublethal injury in the outer membrane (in the LPS molecules) of the cells that allows nisin to pass through the outer membrane and come in contact with the cytoplasmic membrane and destabilize its functions. Recently, Gao et al.(1991) provided evidence that nisin treatment of liposomes, made from *E. coli* phosphatidyl ethanolamine, dissipated membrane potential and the pH gradient and made it permeable to ions. These results thus indicate that Gram-negative bacteria under certain conditions can be made sensitive to nisin.

Nisin Resistance of Gram-positive Bacteria

Several studies have identified developed or spontaneous nisin resistance in Gram-positive bacteria. The survivors of sensitive strains of Gram-positive bacteria could be "trained" to grow in a medium containing as much as 1000-fold higher concentrations of nisin, and these resistant cells did not produce the enzyme nisinase which hydrolyze nisin. However these trained resistant cells lost nisin-resistance after a few transfers in nisin-free medium (Lipinska,1977). Hirsch and Gringsted (1951) used spontaneous resistant mutants of *Lactococcus cremoris* (formerly *Streptococcus cremoris*) to differentiate bacteriocins of different lactococci. Several strains of *L.monocytogenes* also showed spontaneously developed resistance to 500 IU/ml of nisin. *Listeria monocytogenes* Scott A was particularly resistant and was capable of growth at levels of 2000 IU/ml of nisin (Doyle,1988). The mechanism of spontaneous nisin

resistance is not clear.

Nisin-resistant lactic acid bacteria selected for industrial purposes exhibited no cross-resistance with medical antibiotics, such as penicillin, streptomycin, erythromycin, chloramphenicol, tetramycin and aureomycin (Lipinska,1977). As compared with the parent strains, they were much more sensitive to medical antibiotics (Lipinska and Strzalkowska,1964), and more sensitive to adverse factors, such as drastic heating, as well as high salt and bile concentrations (Lipinska, 1975).

Some Gram-positive bacteria have been described to be resistant to nisin due to their ability to synthesize a nisin-hydrolyzing enzyme termed "nisinase". The occurrence of a nisinase from *Lactobacillus plantarum* was reported by Kooy (1952) and was also reported to occur in other lactic acid bacteria (Galesloot,1956; Lipinska, 1977). Further research by Jarvis (1967) showed that the nisinase from several species of *Bacillus* reduced the double bond of dehydroalanyllysine located in the C-terminal of nisin to alanyllysine and this suggested it is a dehydropeptide reductase.

Several studies have been published indicating the existence of a genetic basis of nisin resistance phenotypes (Nis^r). Steen et al. (1991) showed that the gene is encoded in a chromosomally linked transposon. The presence of nisin resistance gene has also been observed in nisin-nonproducing strains of *Lactococcus lactis* (Nis-Nis^r) (Klaenhammer and Sanozky, 1984) and *L. lactis* subsp. *var diacetylactis* (McKay and Baldwin, 1984). In these strains, Nis^r gene

is encoded in plasmids that could be transferred through conjugal mating. Current studies (Forseth and McKay, 1991) have revealed that the nisin-resistant gene is located in a 1.2 Kb DraI fragment and encoded for a 35,035 Dalton protein.

MODE OF ACTION OF NISIN

The exact mechanism of action of nisin at the molecular level against vegetative cells and spores of sensitive Gram-positive bacteria is not entirely understood.

Nisin is a polypeptide with strong cationic properties and a high content of hydrophobic amino acids. Ramseier (1960) suggested that nisin behaves as a surface-active cationic detergent and adsorption to the microbe appears to be the first step required for its action. Recent studies on the mode of action of nisin indicated that the primary target is the cytoplasmic membrane which is disrupted by nisin's interaction with its phospholipid components (Ruhr and Sahl, 1985; Henning et al.,1986; Kordel and Sahl,1986). Interaction of nisin with membranes leads to an efflux of cytoplasmic compounds of low molecular mass and causes a rapid collapse of the membrane potential, resulting in the cessation of biosynthetic activity. The polypeptide requires a transnegative threshold potential for membrane interaction (Sahl et al.,1987). An effect of nisin on cell wall synthesis has also been reported (Ruhr and Sahl,1985; Henning et al.,1986;

Reisinger et al.,1980), which might be a synergistic effect to the disturbance of the membrane function. Recently, Bruno et al. (1992) reported that nisin at the level of 2.5 μg/ml caused the complete dissipation of proton motive force (PMF) of several strains of *L. monocytogenes*.

The outgrowth of bacteria from spores proceeds in three stages: (a) germination swelling, (b) pre-emergency swelling, and (c) emergency and elongation. Unlike other preservatives, nisin is believed to inhibit the germination process at the stage of pre-emergent swelling (Hitchins et al., 1963; Lipinska, 1977). During the following stages, the synthesis of membranes (Dawes and Halvorson,1972) and cell wall components (Chin et al,1968) commences that are specific for the vegetative cells. An inhibition of cell wall synthesis and interference with the membrane function should be detrimental especially to the development of vegetative cells from the spores as, in fact, it is observed with nisin. Most studies suggested that nisin action against spores is sporicidal rather than sporostatic. It has been demonstrated that spores damaged by heat are more sensitive to nisin (Heinemann et al.,1965). Spores of different types of bacteria and even strains of a given species differ in their sensitivity to nisin (Lipinska,1977).

EFFECTIVENESS OF NISIN IN FOOD SYSTEMS

Factors Affecting the Efficacy of Nisin in Foods

Antimicrobial substance, nisin, may be introduced into foods by three different approaches; (1) nisin may be present as a result of the growth of nisin-producing bacteria added as starter cultures, (2) nisin may be introduced as a constituent of the by-products of lactic acid bacteria fermentation (i.e. nisin containing dried milk) into other foods, and (3) nisin may be added as a purified or commercial product (Daeschel, 1990).

The effective application of nisin in foods requires consideration of many factors, including the type of food and the types and numbers of microorganisms which nisin might be required to inhibit. Generally, nisin is most effective in acidic (below pH 6.0) and lowfat foods, where the target organisms are Gram-positive bacteria. Nisin is not effective normally against Gram-negative bacteria, yeasts, molds, and viruses. Also, it is not active against all Gram-positive bacteria, and even for a sensitive strain there can be developed nisin-resistant cells.

As a cationic hydrophobic polypeptide, nisin can be influenced by the environment, food composition and ingredients, and the consistency of a food. Therefore in this section, the effect of food components on nisin activity, enhancement of the efficacy of nisin in certain foods, as well as the successful applications of nisin in foods and beverages are discussed.

Applications of Nisin in Foods

Hirsch et al. (1951) first used nisin as a food preservative. They made Swiss-type cheese with a nisin-producing starter culture which effectively prevented "blowing" faults caused by *C. butyricum* and *C. tyrobutyricum* . Subsequently they used a similar technique for the preservation of processed cheese. The clostridial spores often present in the raw cheese used for processing can survive the heat process of 85-105°C which is achieved during the melting process. The composition of pasteurized processed cheese spread in terms of increased pH and moisture content, and ultimate anaerobiosis favor the outgrowth of spores which may result in subsequent spoilage due to the production of gas and off-flavor and liquefaction of the cheese. Anaerobic spore formers particularly associated with processed cheese are *Clostridium butyricum, Clostridium tyrobutyricum,* and *Clostridium sporogenes* (Meyer,1973; Thomas,1977). Another potential danger of great significance is the growth and formation of toxin of *Clostridium botulinum* in processed cheese spreads.

Nisin was found to be most effective in preventing clostridial spoilage in pasteurized processed cheese (McClintock et al.,1952). Since 1953, nisin has been used experimentally outside of the United States as an antimicrobial preservative in a variety of foods, including cheese and cheese products. Promising results with nisin use has stimulated the manufacture of nisin and a food-preservative grade preparation of nisin is currently marketed.

The research data (Taylor and Somers,1982; 1984) submitted in the GRAS affirmation petition to FDA on the use of nisin showed that nisin prevented the outgrowth of *C. botulinum* spores and toxin formation in the experimental formulations of process cheese spreads at the level of (1) 12.5 ppm, when the salt content was reduced below 1%, and moisture content (50 to 54%) and phosphate emulsifier content (2.5%) were normal; (2) 250 ppm, when the moisture content was above 55%, and the phosphate emulsifier and salt content was reduced below 2.5% and 2%, respectively, and (3) 250 ppm, when the moisture content was normal (50 to 54%), no salt was added, and the phosphate emulsifier content was reduced to 1.7% or less. These studies indicated that the maximum effective concentration of nisin against *C. botulinum* is greater than 100 ppm (5,000 IU/g), but that quantities of nisin 150 ppm (7,500 IU/g) and 250 ppm (12,500 IU/g) are fully effective.

Tanaka et al.(1986) also reported the factors that affect antibotulinal properties in pasteurized processed cheese spreads. Their study indicated what levels of pH, and combined phosphate and salt content were effective in preventing the growth and formation of toxin of *Clostridium botulinum* types A and B in processed cheese spreads with moisture contents ranging from 51 to 60%. The use of nisin of 250 to 500 IU/g, however, allows pasteurized processed cheese spreads to be formulated with high moisture levels and low sodium chloride and phosphate contents (Somers and Taylor,1981). The effective use of nisin can allow processed cheese products to be stored without refrigeration and

negate the risk of spoilage.

According to the above results, the effective range of nisin levels in processed cheese products is 375 to 1250 IU/g (Fowler and Gasson,1990; Anon.,1988).

In processed cheese, nisin is now used in 28 countries including the U.S., UK., Australia, New Zealand and France (Fowler and Gasson, 1990), and it is also permitted for use in the production of canned vegetables and other dairy products in more than 40 countries (Delves-Broughton, 1990).

In canned foods, nisin is used to reduce the energy consumption of sterilizing processes, to improve nutritional value, appearance and texture, because nisin is effective against thermophilic flat-souring types of bacteria and other microflora. Applications of nisin in canned vegetables include potatoes (Lipinska,1977), peas (Vas et al.,1967), mushrooms (Heinemann et al.,1963), and soups (Bardsley, 1962). Nisin also can be used in high acid foods (pH below 4.5) to control acid-tolerant Gram-positive spoilage flora such as *Clostridium pasteurianum* and *Bacillus macerans* in canned tomatoes (Maslennikova et al.,1968). Addition levels of nisin used in canned foods are generally between 100 and 200 IU/g (Delves-Broughton, 1990).

Nisin can also extend the shelf-life and reduce the heat processing of sterilized milk products (Shehata et al.,1976; Wajid and Kalra,1976). Thermophilic bacterial spores can survive commercial sterilization of milk and can cause spoilage if the storage temperature of milk gets to 40°C or higher even

for a short period. Addition of nisin levels from 80 to 100 IU/ml was found to reduce heat processing time by 10 min. as well as to reduce spoilage of canned evaporated milk from the growth of high heat resistant spores during storage (Anon.,1988).

Applications of nisin in the brewing industry are for preventing growth of spoilage lactic acid bacteria in wort. Nisin can also be used to control contamination of "pitching yeast" cultures, reduce pasteurization requirements and increase the shelf-life of unpasteurized or bottle conditioned beers. Nisin does not have any adverse effect on brewing yeasts and effectively suppresses undesirable bacterial growth, resulting in increased fermentation performance (Ogden and Tubb,1985; Ogden,1986; Ogden et al.,1988). Similar applications have been considered in wine production. Nisin at the levels of 2.5 to 25 μg/ml in grape-must reduced the growth of some lactobacilli, and completely eliminated the growth of two other species (*Leu.oenos* and *P. damnosus*), and produced wine with acceptable taste (Radler,1990). Daeschel et al.(1991), furthermore, reported that nisin can control malolactic fermentation (MLF) in wines using nisin resistant *Leuconostoc oenos* strains when the MLF processes are required. Nisin has been shown to have a potential use in other segments of the fermentation industry, such as the production of fruit brandies (Henning et al.,1986b) for preventing unwanted growth of lactic acid bacteria. The use of nisin in the fermentation mash can increase alcohol content in the distillate by over 10%.

Nisin, however, has been reported not to be very effective in meat products and certain high fat containing foods (Scott et al.,1981; Somers and Taylor,1981; Rayman et al.,1983; Taylor and Somers,1985; Jung et al.,1992).

Interactions of Nisin with Food Components

Interactions of nisin with food components may also affect the efficacy of nisin in foods. It was reported that milkfat (Jones, 1974) and phospholipids (Henning et al.,1986) interfered with nisin activity. Jung et al. (1992) observed that the activity of nisin against *Listeria monocytogenes* in fluid milk was directly dependent upon the fat content, with increasing concentrations decreasing the efficacy of nisin. Somers and Taylor (1987) found different residual nisin activities in the cheese spreads throughout the storage period, and nisin levels tended to decrease somewhat with extended incubation at 30°C. They also discussed the possibility that inconsistent extraction of nisin from the cheese emulsion might be caused by nisin binding to the cheese spread ingredients. After nisin is added to a simple or complex matrix, such as a food system, the detectable level of nisin may remain stable or diminish with storage. Rogers and Montville (1991) monitored nisin levels during 35°C storage in 10% albumen, 10% lecithin and 30% soluble starch solution and a basal medium consisting of 0.5% proteose peptone, 0.5% yeast extract, and 1.0% glucose. Nisin levels remained the same for 37 days in all but the egg albumen. Levels of approximately 400 IU/ml nisin dropped to less than 5 IU/ml in less than 48 hrs.

in the 10% albumen solution. At lower initial nisin levels, the outgrowth of either *C. botulinum* or *Lb. sake* in the albumen, starch or lecithin containing media with nisin resulted in progressively decreasing nisin levels over time, and ultimately to zero after approximately three weeks at 35°C. Daeschel et al.(1991) observed that nisin's potential to control malolactic fermentation and to inhibit spoilage bacteria remained stable in white wines for several months, but decreased by up to 90% of the original activity in red wines within 4 months. Further investigation (Bower et al, 1992) showed that tannins in red wine were responsible for the loss of nisin activity in red wine. It has also been reported that the use of nisin for meat preservation has not found much success. Although nisin was quite effective in inhibiting the outgrowth of *C. botulinum* spores in TPYG broth (Scott and Taylor, 1981), nisin was not effective in preventing the outgrowth of *Clostridium* in cooked meat medium at levels of up to 125 ppm (Scott and Taylor, 1981; Somers and Scott, 1981). In bacon, nisin alone had no antibotulinal effectiveness. Nisin alone was not able to inhibit botulinal toxin production in chicken frankfurters and in pork slurries at levels up to 500 ppm (Taylor et al, 1985 and Rayman et al., 1983). The fate of nisin in meat systems is unknown. Several investigators only suggested that poor preservation effect of nisin in meat include the binding of nisin onto meat particles and surfaces, uneven distribution, poor solubility in meat systems, and possible interference with phospholipids (Scott and Taylor, 1981; Bell and De Lacy, 1986; Henning et al.,1986).

Although it is now clear that food composition is an important factor in determining the action of nisin in food systems, not much work has been done on the influence of food components on the efficacy of nisin. Also the mechanism of interaction of nisin with food components is not well described.

Enhancement of the Efficacy of Nisin in Certain Foods

Sodium chloride has been observed to increase the efficacy of bacteriocin against target organisms. Harris et al.(1991) and Okereke and Montville (1991) demonstrated the synergistic effect of NaCl on bacteriocin activity. However, the antagonistic effect of 3% sodium chloride on inhibition of spore outgrowth by nisin was reported by Bell and DeLacy (1985).

Several studies have addressed the influence of food emulsifiers on the activity of nisin. Henning et al.(1986) showed an antagonistic effect of several emulsifiers (mono- and diacylglycerols) on the antimicrobial activity of nisin in tomato juice. However, experiments with Tween 80 in half and half cream system clearly showed an enhancement effect of the emulsifier on nisin activity against *Listeria monocytogenes* (Jung et al.,1992). Blackburn et al.(1989) also demonstrated similar enhancement effects on nisin activity against *Streptococcus agalactiae* and *Listeria monocytogenes* in milk with monoacylglycerols (lauric and oleic acids) which act as non-ionic emulsifiers.

An alternative approach to increase the efficacy and activity spectra of bacteriocin is to make target cells more sensitive by eliminating some of their

natural defensive systems. Blackburn et al. (1989) and Stevens et al. (1991; 1992) reported the effect of a combination of nisin with EDTA to inhibit Gram-negative bacteria which are not ordinarly sensitive to nisin.

HEALTH AND LEGAL ASPECTS

Toxicological Data

Nisin-producing *L.lactis* wild strains occur in milk and in many milk products, hence, nisin is often a natural constituent of dairy products, and has probably been consumed by humans since the initial consumption of milk and milk products.

As for the safety of nisin, studies done in USSR (Lipinska, 1977), Japan (Hara et al.,1962), and England (Frazer et al.,1962) have established its non-toxicity. Commercial British and Russian nisin preparations were examined either alone or in comparison with other food preservatives. Toxicological tests comprised many generations of rats and mice administered normal levels and overdoses of nisin. Results of these experiments consistently confirm the non-toxicity of nisin and slight, if any,effect on some of the properties examined. An in vitro study (Heinmann and Williams, 1966) showed that nisin is degraded by an intestinal enzyme, pancreatin (specifically, ∂-chymotrypsin), suggesting that nisin would not affect the intestinal flora. The authors of this study

hypothesized that nisin is rapidly hydrolyzed and inactivated shortly after it leaves the stomach.

A joint FAO/WHO committee on food additives stated in 1968 that 33,000 IU/Kg of body weight does not present any undesirable effects and is recommended for daily intake (WHO,1969). There are about 37 x 10^6 units in 1 gram of pure nisin. Up to 400 IU/g of food are usually recommended for food preservation, that is about 10 ppm. The average daily intake, 33,000 units, is just less than 1 mg. An acceptable daily intake for a 70 Kg person would thus be about 58 mg of pure nisin.

Acceptance of Nisin as a Food Additive

Nisin has been used commercially outside of United States since 1960. The joint FAO/WHO expert committee accepted nisin in 1969 as a legal food additive, and the Food and Drug Administration approved nisin as a GRAS additive to prevent *Cl. botulinum* in pasteurized cheese spreads in 1988 (Federal Register, April 6, 1988) and permission for use of nisin in a variety of foods in the United States is pending status.

The use of nisin as a food additive is now permitted in more than 40 countries. There are differences in the admissible levels as well as in the products to which it can be added; nevertheless, wide international acceptance is noted (appendix 2).

Chapter 1

Controlling Wine Malolactic Fermentation with Nisin and Nisin-Resistant Strains of *Leuconostoc oenos**

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ABSTRACT

The antimicrobial polypeptide nisin (100 U/ml) prevented malolactic fermentation in wines by indigenous or intentionally added lactic acid bacteria. Nisin resistant (100 U/ml) mutants of *Leuconostoc oenos* were obtained and used with nisin in wine to carry out a pure culture malolactic fermentation in the absence or presence of other lactic acid bacteria. Nisin degradation by mutants was not observed, and residual nisin was detectable in wines 4 months after it was added. Results indicated that nisin or nisin with resistant bacterial starter cultures can be used to control malolactic fermentation in wines.

INTRODUCTION

Malolactic fermentation (MLF) by lactic acid bacteria (LAB) can play an important role (Kunkee,1967) in governing the acidity and sensory characteristics of certain wines. Deacidification is achieved by the conversion of the dicarboxylic malic acid to the monocarboxylic lactic acid. In practice, the pH of wines undergoing MLF will rise 0.1-0.3 pH units. The MLF may be advantageous for some wines, but detrimental for others, depending on the style and composition of wine. Often, the MLF is difficult to manage when it is most needed, such as in wines of low pH, and most difficult to prevent when unwanted, such as in wines of high pH. The MLF can be promoted by inoculation of wine with selected strains of LAB (primarily *Leuconostoc oenos*), which can reduce inconsistencies associated with fermentations conducted by naturally occurring LAB. Growth of the indigenous LAB can lead to spoilage such as ropiness (mucilaginous material) and the formation of excess acetic acid. Suppression of the MLF and the natural LAB can be achieved in some wines by maintaining a low pH (< 3.2), high alcohol content ($> 14\%$) and a high sulfur dioxide level of (> 50 mg/ml). However, these conditions are neither desirable or feasible for the majority of wines.

Recent studies (Radler, 1990a, 1990b) have shown that wine LAB can be inhibited by nisin in both laboratory media and wine musts. The same studies also indicated that nisin did not affect the sensory characteristics of wine. Similar

findings have also been found with nisin in beer (Ogden,1986; Ogden et al., 1985; 1986), in which spoilage LAB were inhibited without affecting flavor. Nisin is a bactericidal peptide produced by *Lactococcus lactis* subsp. *lactis* (Hurst, 1981), which is active against gram-positive bacteria and is used in the food industry to control the growth of *Clostridium botulinum.*

In this study we have investigated the use of nisin to both specifically prevent or promote the MLF by; (1) using nisin to inhibit naturally occurring LAB capable of MLF, and (2) by developing and using nisin resistant mutants of *Leuconostoc oneos* to conduct MLF in wines containing levels of nisin inhibitory to the naturally occurring LAB.

MATERIALS AND METHODS

Microorganisms

Strains of *Leuconostoc oenos* and *Pediococcus pentosaceus* FBB-61-2 were from the authors collection. Strains of *Pediococcus damnosus* were isolated from Oregon produced Pinot Noir wines that were described as spoiled. A commercial yeast preparation (Prise de Mousse, Lalvin EC-1118; Lallemand Inc., Montreal, Canada) was used as inoculum for experimental wine.

Wine

Musts from the1988 harvest (Pinot noir: 21.7 °brix, 10.1 g/L titratable

acidity, pH 3.23, Chardonnay: 22.3 °brix, 10.4 g/L titratable acidity, pH 3.11) were produced from grapes obtained from the Oregon State University vineyard. SO_2 (25 ppm) were added to musts at the time of crush. After the primary yeast fermentation (10 days at 18 °C), the new wines (Pinot Noir; 11.8% alcohol by volume; Chardonnay, 12.5 % alcohol by volume) were racked and frozen in air tight vessels. In some experiments, wine was sterilized by filtration through membrane filters (0.22 μm pore size).

Microbiological Assays

Lactic acid bacteria were cultured and enumerated with a medium (herein called Wine Lactic Acid Bacteria [WLAB] medium) containing 27.5 g/L MRS medium (Difco, Detroit, MI) , 5 g/L fructose, 1 g/L L-malic acid and 10% (v/v) filtered V-8 juice (Campbell Soup Co.). The medium pH (without adjustment) was 5.3 after sterilization (121 °C, 15 min.). Filter sterilized cyclohexamide (50 μg/ml) was added to sterile WLAB to suppress yeast when present. Cultures were incubated at 30 °C under a partial carbon dioxide atmosphere (Gas-pak, BBL Inc.) In wine where both *L. oenos* and *P. damnosus* were added, differential enumeration was done by counting large colonies (> 2 mm in Dia.) as *P. damnosus* and small colonies (< 1 mm in Dia.) as *L. oenos* on WLAB agar spread plates incubated under carbon dioxide at 30 °C for 96 hours. Species differentiation was confirmed by microscopic observation. LAB cultures were added to wines at an inoculum level of $1x10^6$ -

1x10^7 CFU/ml. Growth rates of microorganisms were determined spectrophotometrically (Drew,1981). Nisin resistant mutants (100 Units nisin/ml) of *Leuconostoc oenos* were obtained by step-wise exposure to increasing concentrations of nisin in 5-100 Units/ml steps using WLAB medium. The initial minimum inhibitory concentration of nisin in WLAB for the parental strains was less than 1 U/ml. One mutant, designated *L. oenos* Ey2d-NR1, was used in the experiments with wine.

Nisin and Nisinase Assays

A nisin preparation was obtained from Aplin and Barret Ltd. (Trowbridge, U.K.) that contained 37x106 international units per gram (lot code 201H). A working stock solution of 10,000 Units /ml was prepared by solubilizing the nisin preparation in 0.02 N HCl (~pH 2.0) and storing at 4 °C in the dark. Nisin activity was determined by bioassay using *Pediococcus pentosaceus* FBB-61-2 as the sensitive indicator strain. Bioassay plates were prepared by adding a 0.1% inoculum of a log phase culture of the indicator to 35 ml of tempered WLAB agar that was then poured into petri plates. Wells (5.6 mm Dia.) were made within the agar with a sterile brass cork borer. Standard nisin solutions and nisin containing wine samples were added to wells in volumes up to 100 µl. For enhanced detection of nisin activity , well plates containing samples were allowed to prediffuse for 24 hours at 4 °C prior to incubating at 37 °C for outgrowth of the indicator. Estimates of nisin activity in

samples were obtained by measuring inhibition zone size with a dial micrometer and comparing these with standard linear regression lines($r \geq 0.99$) obtained by plotting the zone diameter versus $\log_{10}$ concentration of nisin.

Nisinase activity was determined as described by Collins-Thompson,et al. (1985) where residual nisin levels were measured in cell-free supernatants from nisin resistant and nisin sensitive *Leuconostoc oenos*. Controls consisted of uninoculated media with and without nisin.

Chemical Analysis

Wines were analyzed for degree brix (soluble solids by refractometer, g /100 g as sucrose), titratable acidity (T.A., g /100 ml as tartaric acid) and alcohol (ebulliometer, vol.%). Malic acid was assayed enzymatically according to the method described by Mc Closkey (1980) and expressed as mg/ml. All expressed concentrations are means of replicate determinations.

RESULTS AND DISCUSSION

Development of nisin-resistant strains

L. oenos Ey2d and Er1a isolated from Oregon wines possess desirable Malolactic fermentation (MLF) abilities under low temperatures and low pHs, and are naturally sensitive to the antimicrobial agent, nisin. Preliminary research has indicated that *L. oenos* will not grow at concentrations of nisin more than 1

IU/ml.

Nisin resistant mutants (100 IU/ml) of *L. oenos* Ey2d and Er1a were obtained by stepwise incremental exposure to increasing concentrations of nisin. Nisin resistant mutants grew approximately 35% slower (Fig.1) as compared to the nisin sensitive parent strains. The observed slower growth rate of nisin-resistant *L. oenos* in WLAB may not be significant in wine since MLFs generally take 1-3 months or longer to be completed.

The chain length of the nisin resistant strains was longer than that of sensitive parent strains. Nisin resistance which is developed, was decreased or lost with several subculture in the nisin-free media. Therefore, the nisin-resistant mutants should be kept in the nisin containing medium to maintain nisin resistance.

Determination of Nisinase Production from *L. oenos*

Nisinase activity is defined in this study as a loss of nisin activity due to degradation or alteration. Nisinase activity was determined using methods described by Collins-Thompson, et al.(1985) where residual nisin levels were measured in cell-free supernatants from nisin resistant and sensitive *L. oenos*. Controls consisted of uninoculated media with and without nisin.

Nisin, a polypeptide is subject to inactivation by certain proteolytic enzymes as well as non-proteolytic enzymes ("nisinase") produced by microorganisms that are resistant to inhibition by nisin. Jarvis and Farr (1971)

characterized an enzyme from *Bacillus cereus* which inactivated nisin by reduction of a dehydroalanine residue adjacent to the C-Terminus. The nisin resistant mutants of *L. oenos* developed and used in this study were tested for ability to inactivate nisin. Significant differences in nisin activity levels were not observed in nisin containing WLAB supernatants in which *L. oenos* resistant mutants or sensitive parent strains were grown. Nor, were any significant differences observed in residual nisin levels in wines inoculated with nisin resistant *L. oenos* and nisin sensitive parent strains. This is an important observation in that it would be counterproductive to have nisin degraded when using nisin resistant mutants to promote a "pure culture" MLF.

Effectiveness of Nisin in Wines

Nisin was able to prevent the growth of either intentionally added LAB capable of MLF or the naturally occurring LAB (Table 1). In the absence of nisin, 2 strains of added *Leuconostoc oenos* were able to grow after an initial decline (at 28 days) to numbers greater than the initial populations. A decline in the inoculum population of *L. oenos* is typically observed (Wibono, et al., 1985) and is believed to be the result of acclimation to the wine environment. At 56 days essentially all malic acid was degraded in treatments with added *L. oenos* but was intact in the same treatments that had nisin added. In addition, at 56 days neither viable LAB or malic acid degradation was evident in noninoculated wines with or without nisin. The growth of the natural LAB in wine is often

variable and is dependent on initial microbial populations, acid and alcohol concentration and availability of nutrients. In our study we did observe growth of the natural LAB at about 5 month's (data not shown) in the absence of nisin but none when nisin was present. Our data are in agreement with those of Radler (1990b) where 100 units nisin per ml in grape must was effective in suppressing the growth of intentionally added *L. oenos* . Thus, nisin may have application to specifically suppress MLF when it is not desirable as well as to suppress bacterial growth which may result in spoilage.

In filter-sterilized wine, intentionally added spoilage LAB (*P. damnosus*) were able to partially degrade malic acid in the wine but were inhibited when nisin was present (Table 2, treatments 2 and 3). In wines with added nisin-resistant *L. oenos* Ey2d-NR1 and parent *L. oenos* (Table 2, Treatments 8 and 10), both strains were able to degrade greater than 90% of the malic acid within 3 months. In the presence of nisin the resistant strains was able to grow and use greater than 95% of malic acid (Treatment 9), whereas the sensitive parent was inhibited (Treatment 11) and malic acid remained intact. When *L. oenos* Ey2d-NR1 was added with *P. damnosus* to wine containing nisin (Treatment 5), only *L. oenos* was able to grow and degrade malic acid. This observation demonstrated that nisin and nisin-resistant strains of desirable MLF bacteria can be used to promote a pure-culture MLF in the presence of other LAB, a situation representative of a commercial winery environment.

The effectiveness of nisin in any foods or beverages is likely to be

dependent on the stability (activity) of the substance over time. A dramatic decrease in nisin activity in Pinot Noir was observed over a 4 month storage period (Fig. 2), but little, if any decrease was observed in Chardonnay (white wine). The presence or absence of added cultures of *L. oenos* to the initially sterile wines did not appear to have an effect on residual nisin activity. It is conceivable that nisin may be interacting with phenolic compounds such as anthocyanins present in red wines, but absent in white wines.

The data presented indicate several potential applications of nisin in commercial winemaking. If desired, MLF may be prevented by inclusion of nisin or may be promoted by its addition and deliberate inoculation of nisin resistant mutants of *L. oenos*. Nisin may also serve to reduce the amount of SO_2 that is used in winemaking to control bacterial spoilage.

Table 1. Inhibitory effect of nisin (100 Units/ml) on the growth of *Leuconostoc oenos* and malolactic fermentation in non-sterile new Pinot Noir wine

Treatment of wine[a]	CFU/ml LAB (mg/ml malic acid remaining)		
	Days after treatment		
	0	28	56
L. oenos Erla	1.1×10^6 (3.7)	2.5×10^4 (2.5)	6.4×10^6(<0.1)
L. oenos Erla + nisin	same as above	< 10 (4.1)	<10 (3.6)
L. oenos Ey2d	same as above	3.4×10^4 (3.2)	1.0×10^7(<0.1)
L. oenos Ey2d + nisin	same as above	<10 (3.9)	<10 (3.4)
nisin	<10 (3.7)	<10 (4.0)	<10 (3.8)
no addition[b]	<10 (3.7)	<10 (ND[c])	<10 (3.8)

a The initial inoculum was 1×10^6 CFU/ml for *L.oenos* Er1a and Ey2d
b LAB were detected (> 1×10^5 CFU/ml) after 5 months
c ND=not determined

Table 2. Inhibitory effect of nisin on *Pediococcus damnosus*, nisin-resistant *Leuconostoc oenos* Ey2d-NR1 and nisin-sensitive *L. oenos* Ey2d and MLF[a]

Treatment		Bacteria CFU/ml		Malic acid
Bacteria added	Nisin added	Pedio.	Leuco.	mg/ml
1. none	no	<10	<10	3.5
2. *P. damnosus*	no	1.6×10^4	<10	1.7
3. *P. damnosus*	yes	<10	<10	3.7
4. *P. damnosus* + *L. oenos* NR1	no	9.0×10^5	5.2×10^7	<0.1
5. *P. damnosus* + *L. oenos* NR1	yes	<10	9.3×10^7	<0.1
6. *P. damnosus* + *L. oenos*	no	1.3×10^6	8.4×10^7	<0.1
7. *P. damnosus* + *L. oenos*	yes	<10	<10	3.2
8. *L. oenos* Ey2d-NR1	no	<10	1.4×10^7	<0.1
9. *L. oenos* Ey2d-NR1	yes	<10	6.8×10^6	0.2
10. *L. oenos* Ey2d	no	<10	7.8×10^7	0.3
11. *L. oenos* Ey2d	yes	<10	<10	3.5

[a] Nisin (100 U/ml) was added to sterile new Pinot Noir wine, which was held at 18°C for 3 months.

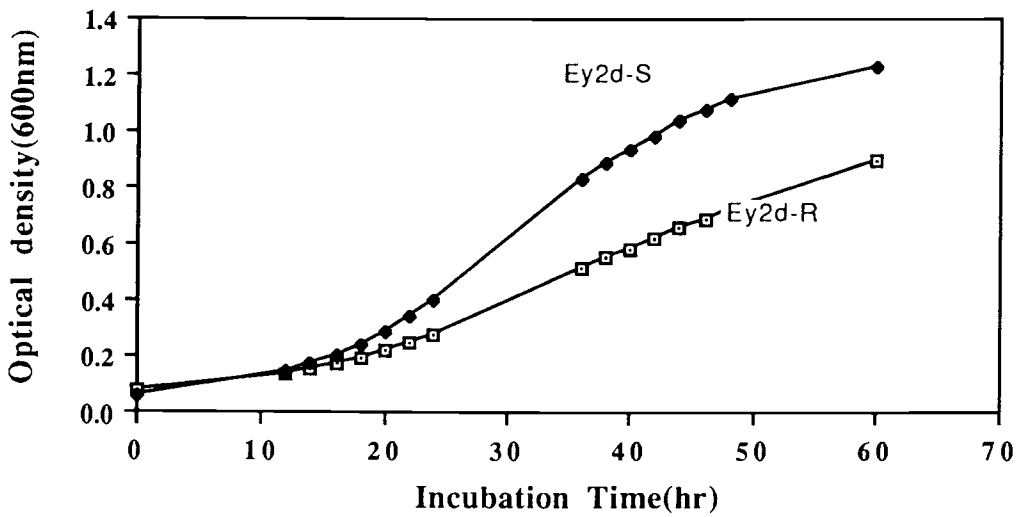

Fig. 1. Growth of nisin-sensitive and nisin-resistant mutants of *Leuconostoc oenos* Ey2d in WLAB at 25°C.

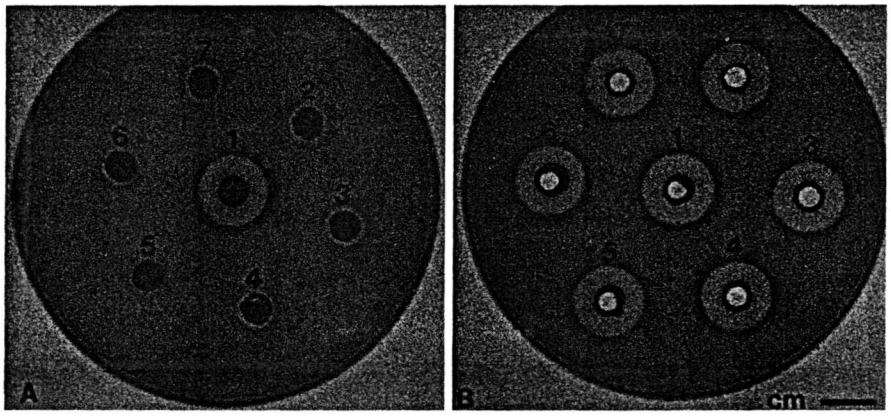

Fig. 2. Residual nisin activity (initial 100 IU/ml) in Pinot Noir (A) and Chardonnay (B) after 4 months storage. Treatments: (1) Control (nisin added to wine just before assay); (2,3) *L. oenos* Ey2d cells added; (4,5) *L. oenos* Er1a cells added; (6,7) no cells added.

Chapter 2

Influence of Fat and Emulsifiers on the Efficacy of Nisin in Inhibiting *Listeria monocytogenes* in Fluid Milks*

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ABSTRACT

The recent FDA affirmation of nisin, an antimicrobial peptide, as a GRAS (generally recognized as safe) additive in pasteurized cheese spreads has renewed interest in its potential application in US dairy products. Fluid milks were prepared with varying concentrations of milk fat (0 to12.9 %) and of nisin (0 to 50 U/ml). Biological activity assays using a sensitive indicator microorganism in a well diffusion system indicated that initial nisin activity (50 U/ml) decreased by about 33% when added to skim milk, and by more than 88% when added to milk containing 12.9% fat. Nisin activity decreased by approx. 50% in milk containing 1.29% fat. Milks containing 0, 10 or 50 U/ml nisin and varying fat levels were challenged with approx. $\log_{10}$ 7 to 7.5 cfu/ml of log phase *Listeria monocytogenes* Scott A or Jalisco. At 2 hr after inoculation the viable count of *L. monocytogenes* Scott A decreased to $\log_{10}$ 0.30 cfu/ml in skim milk with 50 U/ml of nisin, decreased to $\log_{10}$ 2.90 cfu/ml in skim milk with 10 U/ml nisin, and increased slightly ($\log_{10}$ 7.8 cfu/ml) in skim milk without nisin. In half-and-half cream (12.9% milkfat), nisin was far less effective in inhibiting *Listeria* with populations decreasing to $\log_{10}$ 6.57 cfu/ml for 10 U/ml nisin and $\log_{10}$ 5.87 cfu/ml for 50 U/ml. Similar results were obtained with *L. monocytogenes* Jalisco. The non-ionic emulsifier, Tween 80, partially counteracted decreases of nisin activity in milks, whereas the anionic emulsifier, lecithin, had no effect.

Addition of Tween 80 significantly increased the activity of nisin against *L. monocytogenes* in milk regardless of fat content.

INTRODUCTION

Nisin is a member of a group of potent antibacterial substances called bacteriocins. Bacteriocins are proteins or protein containing macromolecules that exert a bactericidal mode of action against susceptible bacteria (33). Nisin, its name derived from "Group N Inhibitory Substance", was first recognized by Rogers and Whittier (28), who initially characterized the inhibitor. More complete characterizations of nisin were provided by Mattick and Hirsch (21). Nisin's application as a food preservative was first evaluated in Swiss cheese (16) in which it effectively prevented blowing (gassing) attributable to the growth of *Clostridia*. Subsequently, nisin's use as a preservative was investigated with a large variety of fresh and processed foods including tomato juice (13), cream-style corn and chow mein (34), meat slurries (26), and more recently with beer (24) and wine (5, 25). Nisin is effective in inhibiting certain gram positive species but not gram negative bacteria, yeasts or fungi. Nisin is also effective in preventing the outgrowth of *Clostridium botulinum* spores (8, 32) which has made it useful in reducing the heating requirement of certain thermally processed foods (3). Recent investigations (1, 12, 22) have indicated that nisin or nisin-producing *lactococci* are inhibitory toward *Listeria monocytogenes*, a foodborne pathogen of concern to the dairy industry. Studies have shown that *L. monocytogenes* is capable of surviving certain manufacturing processes for cheese (30, 31) and non-fat dry milk (9). Most

disturbing observations (29) are that *L. monocytogenes* can grow in fluid milk products held under refrigeration.

After 30 years of safe and efficacious use in many European and Third World countries, nisin was recently affirmed by the Food and Drug Administration (10) as a GRAS (generally recognized as safe) substance for use as an antimicrobial agent to inhibit the outgrowth of *C. botulinum* spores and toxin formation in certain pasteurized cheese spreads. The action by FDA was based on the accumulated body of scientific data indicating that nisin is non-toxic, non-allergenic and is safe and effective as an antimicrobial agent (7, 17).

The ability of an antimicrobial agent to suppress effectively microbial growth or toxin formation is dependent upon a wide range of physical, chemical and biological factors, which include variables such as temperature, acidity, water activity and the types of target microorganisms. Hurst (17), Lipinska (19), and Liu and Hansen (20) have reported on the physical and chemical properties of nisin. Several studies have shown that nisin activity is diminished in foods that contain fat. Jones (18) observed that nisin was more effective in controlling *Staphylococcus aureus* in skim milk than in whole milk and attributed the effect to the milkfat content. In our laboratory, nisin has been observed consistently to be more active on a per unit basis in low fat foods than in high fat foods (4). How fat or lipids interfere with nisin activity may relate to its mode of action. Recent studies (13) indicate that the cytoplasmic membrane

is the major target and is disrupted by nisin's interaction with its phospholipid components. Experiments from the same study demonstrated that various phospholipids antagonized nisin's activity in vitro. In a companion study (14), food emulsifiers (acyl glycerols) were observed also to render nisin much less effective in tomato juice, thus suggesting a limitation to its application in certain foods. However, Blackburn et al.(2) demonstrated an enhanced effect of nisin in milk in the presence of acyl glycerols. The studies presented in this paper demonstrate an enhancement effect on nisin activity in milk by the emulsifier Tween 80. Exactly how lipids and emulsifiers interact with nisin and affect its activity is not clearly understood, but this phenomenon warrants further investigation in order to optimize the effective use of nisin in food and beverage applications.

MATERIALS AND METHODS

Microorganisms

L. monocytogenes strains Scott A and Jalisco (obtained from C. Donnelly, Univ. of Vermont) were cultivated and enumerated with Brain Heart Infusion agar and broth (BBL, Cockeysville MD). Enumeration data represent means of duplicate determinations. *Pediococcus pentosaceus* FBB-61-2 (6) cultivated in MRS broth (Difco, Detroit MI) was used as a sensitive indicator strain in agar well diffusion assays for nisin activity. Incubation temperature was

37 °C for cultivation and enumeration of *Listeria* and for outgrowth of the indicator strain in activity assays.

Milk and Emulsifiers

Skim milk and half-and-half cream were obtained from a local dairy, which obtained its milk supply from a single herd source. The skim and half-and-half were mixed together in 10% increments to give different concentrations of milkfat. Milk fat was determined using the 'Mojonnier Method" (27). Prepared milks (10 ml aliquots) in screw-cap test tubes (16x150 mm) were treated by heating them under flowing steam (100 °C) for 10 minutes. Stock solution of nisin was added to give a final concentration of 0 to 100 U/ml. Thirty minutes after nisin addition, log phase cells of *L. monocytogenes* were added and held at 37 ^{0}C for 2 hr prior to enumeration. Emulsifiers used were polyoxyethylene sorbitan monooleate [Tween 80 or Polysorbate 80] (Sigma, St. Louis,MO) and Soybean Lecithin (ICN Biochemicals, Costa Mesa, CA).

Nisin and Activity Assay

High potency grade nisin was obtained from Alplin and Barrett, Ltd. (Dorset, UK). Activity was indicated as 37x10^6 U/g (Lot Code 201H). Stock solutions (10,000 U/ml) were prepared by solubilizing in distilled water acidified to pH 2 with hydrochloric acid. Five milliliter aliquots were kept frozen at -80 °C until use. Activity assay for nisin consisted of preparing 150x25 mm MRS

agar plates seeded with a 0.1%(vol/vol) of a standardized overnight culture of the indicator strain. Wells of 5.6 mm diameter were made in the solidified agar with a sterilized cork borer. Duplicate samples containing nisin were added in 100 µl volumes to each well. Plates containing samples were allowed to diffuse for 24 hours at 4 °C prior to incubation at 37 °C for outgrowth of the indicator. Estimates of nisin activity in samples were obtained by measuring inhibition zone widths (the distance from the edge of the inhibition zone to the edge of the well) with a dial micrometer and comparing these with linear regression lines ($r>0.98$) obtained by plotting the square of zone width versus $\log_{10}$ concentration of nisin (15).

RESULTS AND DISCUSSION

The results presented herein demonstrated that the sensitivity of *L. monocytogenes* to nisin in milk was strongly dependent on the milk fat concentration. Figure 1 shows the results of an experiment designed to evaluate the inhibitory effects of nisin against *L. monocytogenes*. A concentration of 50 U/ml of nisin was very effective in reducing the initial population (5 to $7x10^8$ cfu/ml) of *L. monocytogenes* Scott A (4 to 6 log reduction) in Brain Heart Infusion broth and non-fat skim milk. However, nisin was much less effective (< 1 log reduction) in half-and-half at concentrations of either 10 or 50 U/ml.

The same effect is illustrated with Figure 2. In this experiment, milks

were prepared with varying concentrations of milk fat (0 to 12.9%) and of nisin (0 to 50 U/ml). After a 2 hr incubation at 37 °C, the viable count of *Listeria monocytogenes* Scott A in skim milk with 50 U/ml of nisin decreased from $\log_{10}$ 7.53 to $\log_{10}$ 0.3 cfu/ml, whereas in half-and-half (12.9% milk fat) the population of *Listeria* decreased only to $\log_{10}$ 5.87 cfu/ml. The same general effect of milk fat on nisin activity was observed when 10 U/ml of nisin was used. Identical experiments (Figure 2) were conducted using *L. monocytogenes* Jalisco. Essentially results were the same as those seen with the Scott A strain, except nisin was less inhibitory at the higher (> 6%) milk fat percentages.

The residual nisin activity in milks that contained 50 U/ml of nisin and varying concentrations of milk fat (0 to12.9%) were determined (Figure 3) by agar well diffusion assay using *Pediococcus pentosaceus* FBB-61-2 as a sensitive indicator organism. The size of the inhibition zone was dependent on the concentration of active nisin in each sample. The dark inner bands represented milk solids of low diffusion mobility. As milk fat concentration increased, less nisin activity was noted. An effect of milk components other than fat on nisin activity also can be seen when comparing the control well with the non-fat skim milk well. It may be possible that milk proteins can bind or interact somehow with nisin. Reduction of nisin activity in the presence of meat proteins was observed by Scott and Taylor (32). This pattern also was shown in Figure 1. Estimates of nisin activity by comparison of the standard curve indicated that, as milk fat content increased, the residual percent activity of nisin decreased

sharply. A 33 % decrease was observed in skim milk and a greater than 88 % decrease in half-and-half.

Therefore, it is evident that milk fat has a significant effect on the activity of nisin and on the ability of nisin to inhibit *L. monocytogenes* in fluid milk. Exactly how milk fat interferes with nisin is not understood. Although the preceding results suggest limitations to the application of nisin in fat containing dairy foods, the following experiments indicated approaches to overcome possible limitations.

If the decreased antimicrobial effectiveness of nisin in half-and-half is attributed to the binding of nisin to the milk fat globule, perhaps this binding could be prevented by adding certain kinds of emulsifiers, which function to reduce surface tension effects that are elicited between polar and nonpolar molecules. To investigate the effect of emulsifiers on nisin activity in milk, we initially tested a range of emulsifier concentrations (0.05 to 10% (vol/vol) for Tween 80 and 0.1 to 0.3% (wt/vol) for lecithin) into half-and-half and skim milk. It was observed that lecithin had little effect, regardless of concentration, whereas the effect of Tween 80 did not increase beyond a concentration of 0.2%(vol/vol). The results from agar diffusion assays showed that Tween 80 does partially prevents loss of nisin activity in half-and-half (Table1). Half-and-half retained 19.6% of added nisin activity; with Tween 80, it retained 43.4% of the original activity. However, lecithin, which is an anionic lipophilic emulsifier, had no effect on nisin activity in half-and-half. Nisin may adsorb to milk fat

globules, which may render it unavailable to destroy microbial cells. Tween 80 has the ability to displace proteins from the milk fat globule (23). Such a phenomenen may occur with nisin if it is adsorbed to the milk fat globule, hence, perhaps explaining the observation of restored or retained nisin activity in milks when Tween 80 is added. Electron microscopy observations reported by Goff et al.(11) showed that Tween 80 decreased by half the number of casein micelles adsorbed to milk fat globules as well as the total amount of protein adsorbed. Extending the experiment described above (Table 1), we tested the effect of Tween 80 on nisin activity in inhibiting *L. monocytogenes* in milk that contained varying amounts of milk fat and a constant amount of nisin (50 U/ml). At 2 hr after inoculation with approximately $\log_{10}$ 6.34 and 7.60 cfu/ml of *L. monocytogenes* Scott A and Jalisco respectively, the viable count in half-and-half with Tween 80 decreased to $\log_{10}$ 2.0 cfu/ml for Scott A and to $\log_{10}$ 1.52 cfu/ml for Jalisco (Figure 4). This was in sharp contrast to treatments not receiving Tween 80, for which nisin activity was significantly reduced.

Observations reported by Henning et al.(14) showed an antagonistic effect of emulsifiers on the antimicrobial activity of nisin in a tomato juice system. Our experiments with Tween 80 clearly showed an enhancement effect of the emulsifier on nisin activity in high fat milks whereas neither an enhancement nor an antagonitic effect was seen with lecithin. Our data are in agreement with those of Blackburn et al. (2), who demonstrated similar nisin enhancement effects against *Streptococcus agalactiae* and *Listeria monocytogenes* in milk with

monoacylglycerols (lauric and oleic acids), which function as non-ionic emulsifiers. The information presented in this study may be useful for dairy food processors considering the use of nisin as an antimicrobial agent in their products. Obvious factors to consider are the fat and emulsifer content of the product because they will likely influence nisin activity.

It is apparent from this study and others that the activity of nisin in foods is strongly dependent on the chemical composition of the particular food to which it added. It appears likely that binding or adsorbtion of the polypeptide structure of nisin occurs with certain food components which makes it inactive or unavailable to inhibit microorganisms. The nature of these binding or adsorption events is a focus of current investigations.

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TABLE 1. Effect of emulsifiers on nisin activity in half-and-half cream and skim milk [1]

Sample treatment [2]	Inhibition zone dia. (mm)	% nisin activity
skim milk	24.0	82.7
skim milk+Tween 80	24.8	100
skim milk+lecithin	23.8	78.9
half-and-half cream (h+h)	16.2	19.6
(h+h)+Tween 80	21.0	43.4
(h+h)+ lecithin	16.0	19.1
nisin 50 U/ml	24.8	100
controls (no nisin)		
skim milk	0	NA [3]
(h+h)	0	NA
Tween 80	0	NA
lecithin	0	NA

[1] Tween 80 was added at 0.2% (vol/vol) and lecithin at 0.2% wt/vol.

[2] All contain 50 U/ml of nisin.

[3] NA=not applicable.

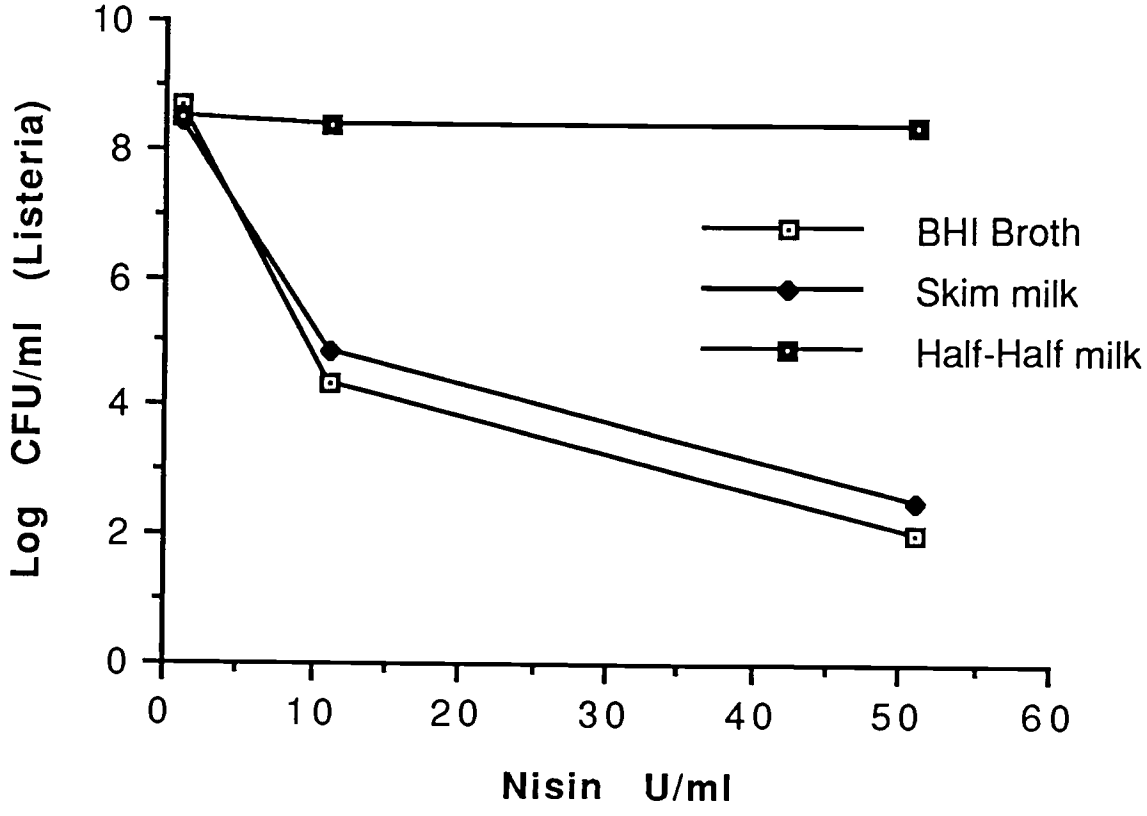

Figure 1. Effect of nisin on survival of *Listeria monocytogenes* Scott A in different media. Cells were exposed to nisin for 2 hours at 37 °C prior to enumeration. Half-and-half contained 12.9% milkfat.

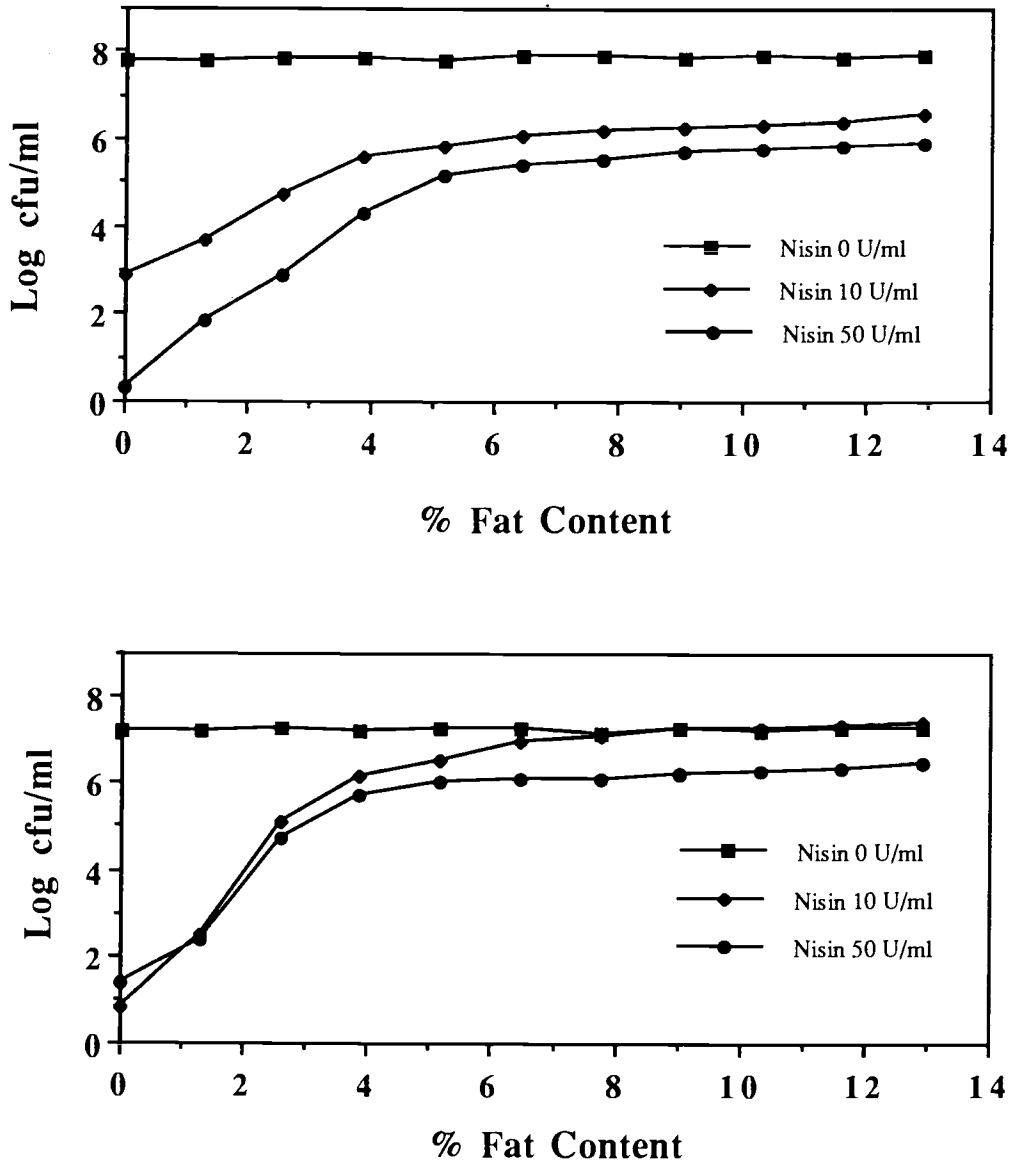

Figure 2. Effect of milk fat content of fluid milk on the ability of nisin to inhibit *Listeria monocytogenes*. Sterile milks containing 0, 10, and 50 U/ml of nisin were inoculated with *L. monocytogenes* Scott A (top) at $\log_{10}$ 7.53 cfu/ml for 0 U/ml, 7.34 for 10 U/ml, and 7.25 for 50 U/ml of nisin and with *L. monocytogenes* Jalisco (bottom) at $\log_{10}$ 7.15 cfu/ml for 0 U/ml, 7.57 for 10 U/ml and 7.25 for 50 U/ml of nisin.

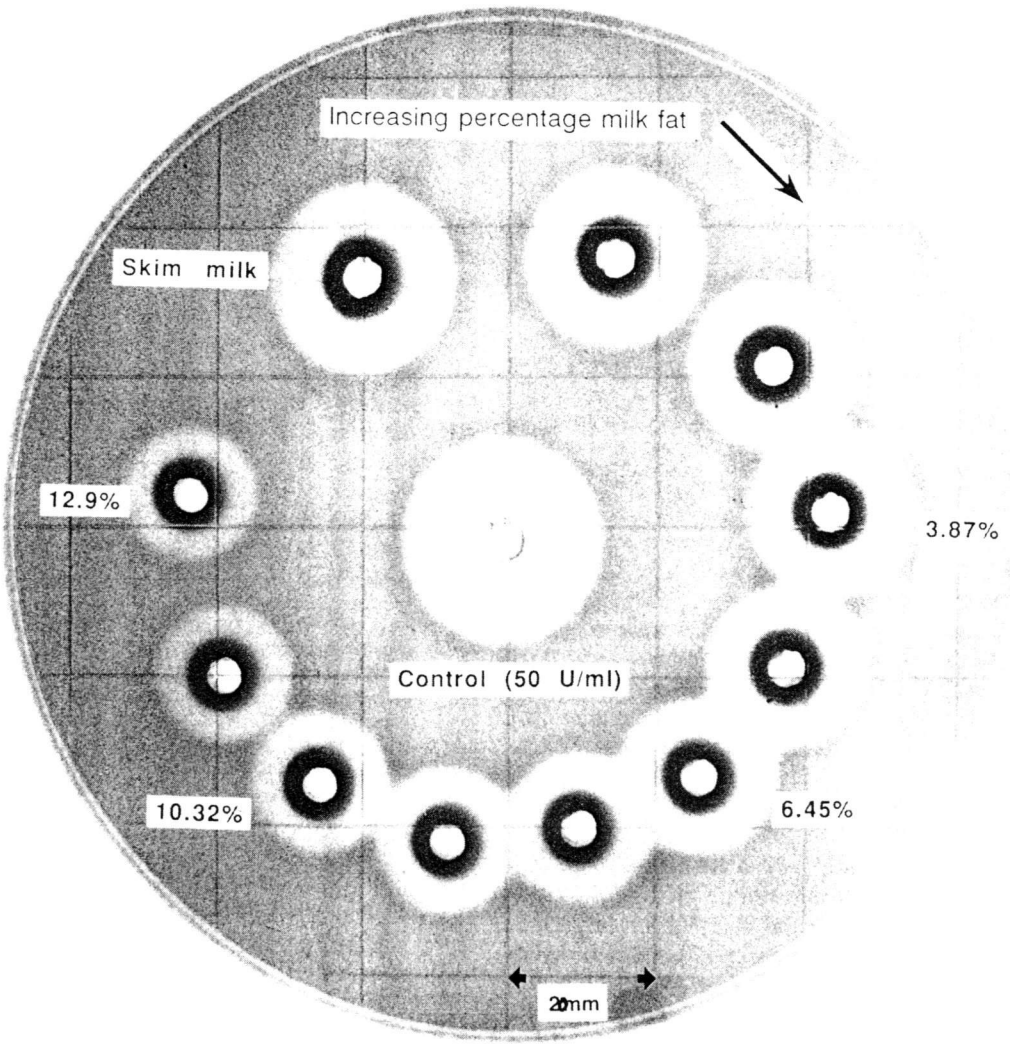

Figure 3. Effect of milk fat concentration in milk on nisin activity (50 U/ml) in agar diffusion assays. Half-and-half cream was diluted with skim milk in 10% increments to give varying concentrations of milk fat.

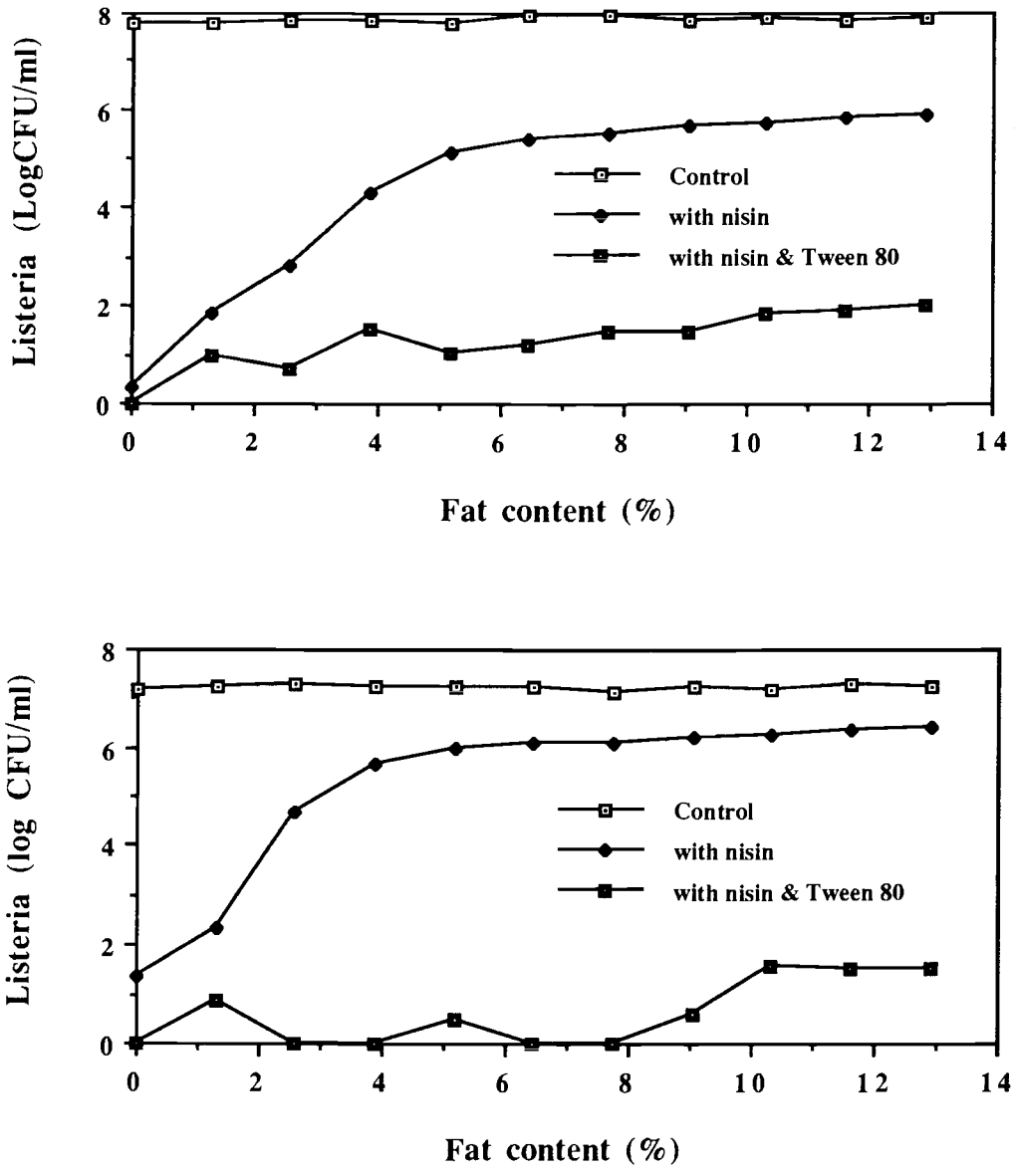

Figure 4. Effect of Tween 80 (0.2% vol/vol) on the efficacy of nisin (50 U/ml) in milks with varying fat content. Sterile milks were inoculated with *Listeria monocytogenes* Scott A (top) at $\log_{10}$ 7.53 cfu/ml for treatments without Tween 80 and $\log_{10}$ 6.34 with Tween 80 and with *L. monocytogenes* Jalisco (bottom) at $\log_{10}$ 7.20 cfu/ml for treatments without Tween 80 and $\log_{10}$ 7.60 cfu/ml with Tween 80.

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Chapter 3

Effect of Interaction of Nisin with Milk Lipid Components on the Efficacy of Nisin

ABSTRACT

The antimicrobial peptide, nisin was exposed to various fatty acids, triacylglycerols and phospholipids to determine their influence on the efficacy of nisin in high fat containing food systems and to identify the nature of interaction of nisin with these components. Nisin activity was quantified by agar well diffusion bioassay method and expressed as percent of original activity. In the previous experiments (J. Dairy Sci. 75:387, 1992), it was observed that nisin activity in fluid milks was directly dependent upon milk fat content with increasing concentrations decreasing the efficacy of nisin. However, isolated milkfat (triacylglycerols) from half-and-half cream did not interfere with nisin activity when reconstituted into skim milk at various concentrations of milkfat. Although nisin in half-and-half remained less than 20% of its activity, nisin in pH adjusted half-and-half (pH 4.6) retained more than 90% of nisin activity. These results suggest that triacylglycerols may not be responsible for the loss of nisin activity in fluid milk, and that lowering the pH of milk may cause certain milk components to change thier binding properties to nisin molecules. Major triacylglycerol components of milkfat tested did not exhibit an antagonistic effect against nisin activity. Phosphatidyl choline (PC), phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS) at the concentrations of 0.5 mg/ml reduced nisin activity by 6%, 11.7% and 58.9% of original activity, respectively. When these phospholipids were combined with triacylglycerol, they elicited much greater

antagonistic effect against nisin. When PC, PE or PS was combined with a mixture of triacylglycerols, nisin activity was diminished by 38%, 62% and 83% of original activity. Fatty acids also interfered with nisin activity with methylated fatty acids being less influential than unmethylated fatty acids. Nisin activity decreased by 19.2% with methylated oleic acid ($C_{18:1}$) and decreased by 65% with unmethylated oleic acid ($C_{18:1}$).

INTRODUCTION

Nisin, synthesized and secreted by certain strains of *Lactococcus lactis*, is the first Lactic Acid Bacteria (LAB) bacteriocin identified and applied in food preservation. It is very effective against Gram-positive and spore forming bacteria and foodborne pathogens such as *Listeria monocytogenes*. Nisin is, at present, the only bacteriocin approved as a GRAS additive by the FDA (Federal Register April 6, 1988), specifically to prevent *Clostridium botulinum* growth and subsequent toxin formation in pasteurized processed cheese spreads.

However, nisin's low solubility (Hurst,1981), heat sensitivity and decreased efficacy (Tramer,1964) at above neutral pH has limited its applications in a variety of foods. Generally, nisin is most effective in acidic foods. Rayman et al.(1981) showed that the effectiveness of nisin decreased with increasing pH.

Interactions of nisin with food components also may affect the efficacy of nisin in foods. It was reported that milkfat (Jones,1974) and phospholipids (Henning et al.,1986) interfered with nisin activity. Jung et al.(1992) observed that the activity of nisin against *Listeria monocytogenes* in fluid milk was directly dependent upon the fat content with increasing concentrations decreasing the efficacy of nisin. Somers and Taylor(1987) found different residual nisin activities in the cheese spreads. They discussed the possibility that inconsistent extraction of nisin from cheese emulsion might be caused by nisin binding to the cheese spread ingredients. Daeschel et al.(1991) observed that nisin's potential to

control malolactic fermentation and to inhibit spoilage bacteria was retained stable in white wines for several months but decreased up to 90% of original activity in red wines within 4 months, and further investigation (Bower et al., 1992) showed that polymerized polyphenolics (tannins) in red wine were responsible for the loss of nisin activity in red wine. It has also been reported that the use of nisin for meat preservation has not found much success. Nisin alone was not able to inhibit botulinal toxin production in chicken frankfurters and in pork slurries at levels up to 500 ppm (Taylor et al, 1985 and Rayman et al.,1983). Nisin was ineffective in preventing the outgrowth of *Clostridium botulinum* spores in cooked meat medium at levels of up to 125 ppm (Scott and Taylor,1981 and Somers and Taylor,1981). The fate of nisin in meat systems is unknown. Several investigators suggested that the poor preservation effect of nisin in meat may include the binding of nisin onto meat particles and surfaces, uneven distribution, poor solubility in meat systems (Scott A and Taylor,1981), and possible interference by phospholipids (Henning et al.,1986). Although it is now clear that food composition is an important factor in determining the efficacy of nisin in food systems, few studies have addressed the influence of food components or other ingredients on the activity of nisin. In addition, the mechanisms of interactions of nisin with food components is not understood and warrants investigation.

To determine which food components are responsible for the loss of nisin efficacy in certain foods and to identify the mechanism of interaction of nisin

with these components, several saturated and unsaturated fatty acids, triacylglycerols and phospholipids as major milk lipid components were evaluated for their ability to influence the efficacy of nisin in food systems.

MATERIALS AND METHODS

Milk and Milkfat

Skim milk and half-and-half were obtained from a local dairy. The pH of Half-and-half with or without nisin was adjusted to pH 4.6 using 0.1N HCl. Both half-and-half and pH adjusted half-and-half were centrifuged or filtrated using Whatman #2 paper to remove cream portions and measured nisin activity in the supernatants or filtrate. Milkfat was isolated using the Majonnier method (Richardson, 1985), and added into skim milk to obtain milks with varying lipid content.

Lipids

Triacylglycerols (tributyrin, tricaproin, tricaprin, trimyristin, tripalmitin, tristearin and triolein), L-∂-phosphatidyl-L-serine (from bovine brain), and L-∂-phosphatidylethanolamine (from sheep brain) were purchased from Sigma Chemical Co.(Louis, MO) and Phosphatidylcholine (soybean Lecithin) were obtained from ICN Biochemicals (Cleveland, OH).

Stock solutions of triacylglycerol(TG) at 0.1M, and of phosphatidyl

choline and phosphatidyl ethanolamine at 0.1g/ml were prepared in chloroform and kept frozen until use. Lipids (triacylglycerol or phospholipid) were added to 2 ml of distilled water contained 150 IU/ml of nisin to measure the effect of TG or phospholipid on nisin activity. Each sample was mixed thoroughly and kept at 4°C for 1 hour and then evaluated for the residual nisin activity. Same amount of solvent (chloroform) was used instead of lipid in every step as a control and subtracted the effect of solvent on nisin activity from each sample. Mixed TG was prepared by mixing 10 µl of each TG into 2 ml of distilled water. Mixtures of TG and phospholipid were prepared by mixing 10 µl of TG and 10 µl of phospholipid in 2 ml of distilled water.

Fatty acids (saturated, unsaturated and methylated fatty acids) were purchased from Sigma Chemical Co.(Louis, MO). Stock solutions of 0.01M of fatty acid were prepared with absolute ethylalcohol and kept frozen at -20°C until use. Nisin(100 IU/ml) was exposed to 200 µl of fatty acid in 5ml of distilled water and measured the residual activity after 1 hr reaction time at 4°C. Each experiment was conducted as duplicate and absolute ethylalchol was used as a control.

Nisin and Activity Assay

High potency grade nisin was obtained from Alplin and Barrett, Ltd. (Trowbridge, U.K.) which contained 37 x 10^6 IU/g (lot code 201H). Stock solutions(10,000 IU/ml) were prepared by solubilizing in distilled water

acidified to pH 2 with hydrochloric acid and kept frozen at -80°C until use. Nisin activity was determined by agar well diffusion bioassay as previously described (Jung et al.,1992). *Pediococcus pentosaceus* FBB-61-2 cultivated in MRS broth (Difco, Detroit, MI) was used as a sensitive indicator strain in agar well diffusion assays for nisin activity. Estimates of nisin activity in samples were obtained by measuring inhibition zone size with a dial micrometer and comparing these with standard linear regression lines($r^2 \geq 0.95$) obtained by plotting the zone diameter versus $\log_{10}$ concentration of nisin.

RESULTS AND DISCUSSION

Jung et al.(1992) observed that milk fat has significant antagonistic effect against nisin activity. However, how exactly fat interferes with nisin activity was not clear. In order to determine the nature of interaction of nisin with milkfat, major components of milk lipid were evaluated for thier effect on nisin activity.

Milkfat was isolated from half-and-half using the Majonnier method (Richardson, 1985) and reconstituted into skim milk to obtain final concentrations of milk fat from 2% to 40%. No significant differences in residual nisin activity level were noted in reconstituted milks with various concentrations of milkfat (Fig.1). These results indicate that triacylglycerols (TG) might not be responsible for the loss of nisin activity in fluid milk.

Milk lipid consists of 98% of TG, 1% of phospholipid and 0.5% of

lipoprotein particles (Walstra and Jenness, 1983). For reconstituted milks, the substances of the natural fat globule membranes are largely absent, and the entire fat surface is covered with plasma proteins (Mulder and Walstra, 1974). If phospholipid, rather than TG, has a significant effect on nisin activity by electrostatically binding to cationic nisin molecules, then in theory, phospholipid in milk would likely lose its binding properties to nisin as lowering pH of milk.

Half-and-half cream (12.9% fat) and pH adjusted half-and-half (pH 4.6), both containing nisin were centrifuged or filtered to remove fat portions, and the residual nisin activity determined in the supernatant or filtrate. Supernatants of half-and-half (whey or plasma milk) retained less than 20% of original nisin activity. However, supernatant and filtrate of the pH adjusted products retained more than 90% of nisin activity (Fig. 2), which is greater than in skim milk (pH 6.6). Reducing the pH of milk probably causes changes in the properties of milk components as well as increases in activity due to the effect of pH on nisin. We propose that, as the pH of milk is lowered, phospholipids of milk lose the negative charges of phospholipids, resulting in a reduction of the ability to bind positively charged nisin molecules.

Major phospholipids of milk, phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine, are associated with the fat globule membrane (Webb et al., 1974). About one-third of the milk phospholipids in freshly drawn milk is located in the milk serum as milk fat globule membrane (MFGM), and their proportion in milk serum can be increased in processed milk

as a result of disruption of the MFGM and release of membrane phospholipids into the aqueous phase (Mulder and Walstra, 1974). Most of the phospholipids associated with cream are recovered in the aqueous phase (buttermilk) after churning. Thus cream portion has far less phospholipids. About 50% of the phospholipids of whole milk are found in skim milk, probably as components of small fat globules and lipoprotein particles (Webb, et al.,1974).

It is, therefore possible that if interaction of nisin with phospholipids in milk rather than triacylglycerols causes reduction of nisin activity, phospholipids in skim milk may be responsible for the slightly decreased nisin activity in skim milk. It may also explain why nisin is effective in certain high fat containing foods such as processed cheese spreads which retained less phospholipids content compared to fluid milk.

Figure 3 illustrates the effect of triacylglycerols and phospholipids on nisin activity. Little effect was observed with the major triacylglycerols of milk fat on nisin activity as represented as the control values in Figure 3. Phosphatidyl serine, phosphatidyl choline and phosphatidyl ethanolamine at the concentration of 0.5 mg/ml retained nisin activity 58.9%, 94% and 88.3%, respectively. Interestingly, each triacylglycerol exhibited a much greater antagonistic effect on nisin activity when it was combined with phospholipid. Especially, with phosphatidyl serine which has one more negative charge on its head group, triacylglycerols retained nisin activity of only 20% to 40% of original activity, depending upon triacylglycerols tested. Among the triacylglycerols tested,

tricaproin ($C_{6:0}$) and tricaprin ($C_{10:0}$) combined with phospholipids showed significant antagonistic effect against nisin. On the other hand, trimyristin ($C_{14:0}$) and tripalmitin ($C_{16:0}$) had less effect on nisin activity when they were combined with phospholipids. Mixed triacylglycerols did not give an antagonistic effect against nisin activity. When a mixture of triacylglycerols was combined with phosphatidyl serine, phosphatidyl choline and phosphatidyl ethanolamine at concentrations of 0.5 mg/ml, the residual nisin activity were about 17%, 62% and 38%, respectively.

Saturated, unsaturated and methylated fatty acids were tested to determine their interactions with nisin. Among saturated fatty acids tested, palmitic acid ($C_{16:0}$) exhibited the highest interaction with nisin, resulting in the least nisin activity remaining (Fig. 4). Methylated fatty acids did not diminish nisin activity as much as unmethylated fatty acids did. Methylated oleic acid ($C_{18:1}$) retained nisin activity 80.8%, while unmethylated $C_{18:1}$ retained only 35% of original nisin activity.

Therefore, it was concluded from this study that nisin interacts much more with negatively charged phospholipids rather than neutral triacylglycerols. Free fatty acids also gave an antagonistic effect against nisin. Electrostatic interaction between the negatively charged head group of the phospholipids or lipids and the positively charged nisin molecules, is the likely interaction of nisin with food components which renders nisin activity lost in the food sysems.

Table 1. The effect of free fatty acids and methylated fatty acids on nisin activity

fatty acid	residual activity(%)
Palmitic acid($C_{16:0}$)	37.7
Stearic acid($C_{18:0}$)	43.9
Oleic acid($C_{18:1}$)	34.9
*Me - $C_{16:0}$	60.8
*Me - $C_{18:0}$	65.8
*Me - $C_{18:1}$	80.8

* methylated fatty acid

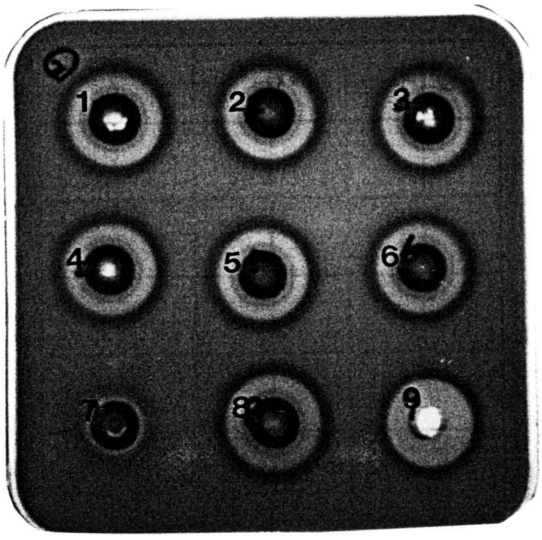


Fig. 1. Residual nisin activity (100 IU/ml) in reconstituted milks with isolated milkfat content from 2 to 40%. Treatments: (1) 2%; (2) 5%; (3)10%; (4) 15%; (5) 20%; (6) 40% milkfat contained; (7) nisin in Half-Half milk; (8) nisin in skim milk; (9) nisin in 0.02N HCl.

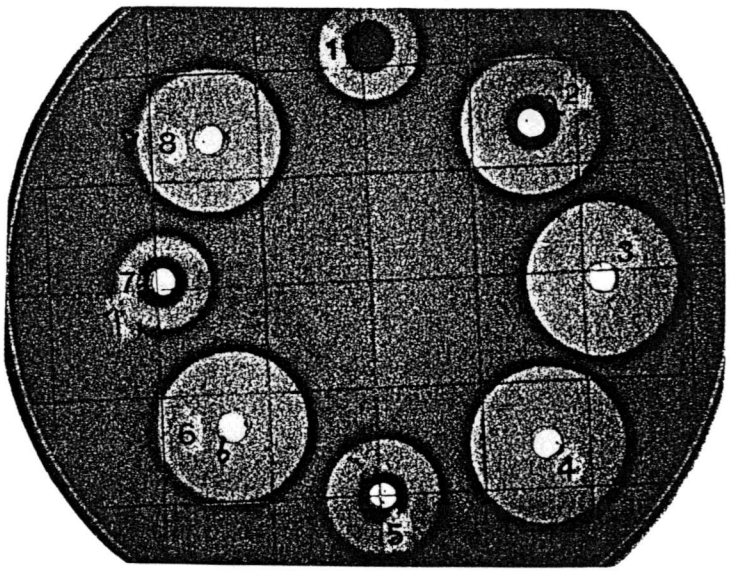


Fig. 2. The effect of pH of half-and-half cream (h+h) on nisin activity. Treatments: 1, 5 and 7 (supernatants), nisin in h+h; 3 (supernatant), 4 (filtrate) and 6, nisin in pH adjusted h+h; 2, nisin in skim milk; 8, nisin in 0.02 N HCl

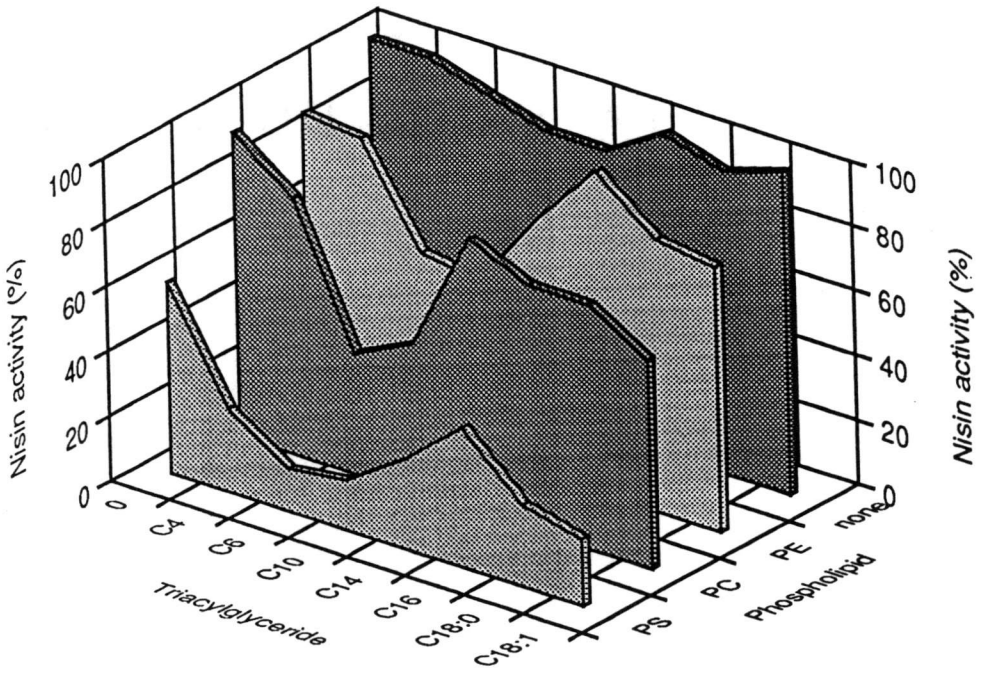


Fig. 3. The effect of triacylglycerols and phospholipids on nisin activity. None: Control (triacylglycerol without phospholipid), PE: phosphatidyl ethanolamine, PC: phosphatidyl choline, PS: phosphatidyl serine.

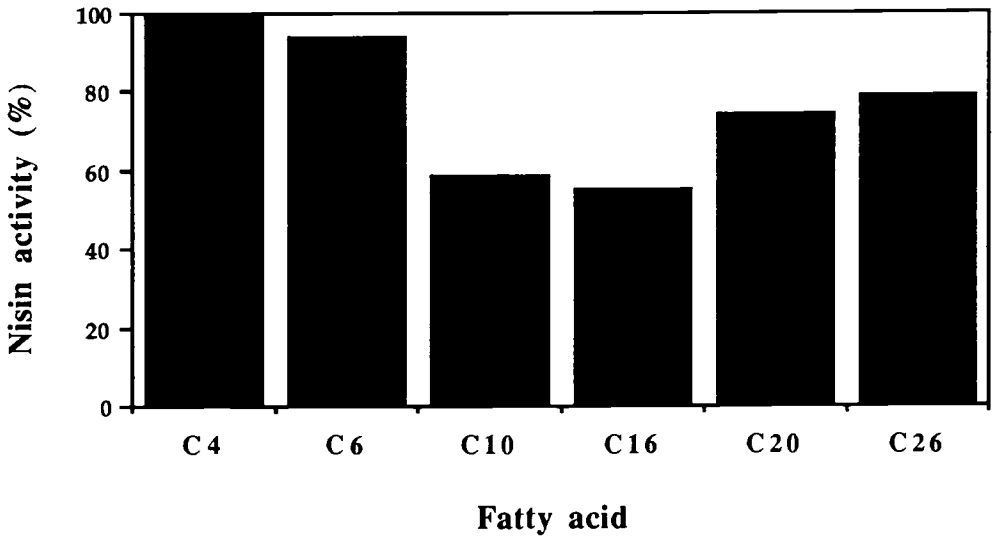


Fig. 4. Effect of fatty acids with different chain length on nisin activity.

Chapter 4

Enhancement of Food Preservation Effect of Nisin by Divalent Cations in Certain Foods and Beverages

ABSTRACT

Nisin has been observed to be less active as an antimicrobial agent in certain high fat containing foods. To investigate the enhancement effect of the reduced efficacy of nisin in certain foods, cations were added into model foods which retained less nisin activity. Residual nisin activity in food systems was measured by agar well diffusion bioassay using *Pediococcus pentosaceus* FBB-61-2 as a sensitive indicator organism. Calcium ions enhanced nisin activity in half-and-half cream, red wine and high fat containing foods. The enhancement effect of calcium ions on nisin activity was varied with different media and concentrations used. With 2% of calcium chloride, nisin activity was 120.8% in phosphate buffer, 151.6% in skim milk and 46.9% in half-and-half. Sodium chloride at 1% did not give an enhancement effect, but rather slightly decreased nisin activity in Half-Half milk. Nisin activity was decreased significantly in high fat containing foods such as beef (5.63% fat), ham (8.45% fat), chicken (8.45% fat), and turkey (7.04% fat). However, nisin with calcium ions or magnesium ions retained much higher activity in these foods. The recovery effect of magnesium ions on nisin activity in food systems was intermediate between that of calcium ions and sodium ions. We believe that the mechanism of enhancement of nisin activity is likely by masking negatively charged sites in the food components which might bind to positively charged nisin molecules by metal ions.

INTRODUCTION

Nisin is an antimicrobial peptide produced by certain strains of *Lactococcus lactis*. Nisin is effective in inhibiting Gram positive and spore forming bacteria and foodborne pathogens such as *Listeria monocytogenes*, but not Gram negative bacteria, fungi or yeasts.

Nisin is used extensively in European and other countries as a preservative in dairy products, vegetables, soups and sauces. In the United State, nisin was recently approved as a GRAS additive by the FDA (Federal Register, April 6, 1988) to inhibit *C. botulinum* and its spores in pasteurized processed cheese spreads.

Nisin as a food preservative has attracted a number of researchers to look for potential applications. Nisin is the best studied and at present the only LAB bacteriocin granted GRAS status for limited applications as a food additive in the USA.

However, several observations of decreased nisin activity in food systems have been reported in the literature. Jones (1974) observed that nisin was more effective in controlling *Staphylococcus aureus* in skim milk than in whole milk. Recently, Jung et al.(1992) reported that milk fat displayed an antagonistic effect against nisin to inhibit *Listeria monocytogenes* in fluid milk. Daeschel (1990) demonstrated that nisin more markedly lost its initial activity in high fat foods (Turkey puree, 7.8% fat) than in low fat foods (green bean puree, 0.2% fat).

Daeschel et al.(1991) also observed that the potential of nisin to control malolactic fermentation and to inhibit spoilage bacteria remained stable for several months in white wine, but lost up to 90% activity within 4 months in red wines. Further research (Bower et al, 1992) has shown that tannins in red wine are responsible for loss of nisin activity in this beverage system. Since the realization of the potentially harmful effects of nitrite, which is used for preservation of meats, there has been an increasing interest in the possible use of nisin as an alternative to nitrite in meat products. However, it has been reported that nisin is not very effective in meat products. Reasons for the poor preservation effects of nisin in meats is not understood.

Sodium chloride has been observed to increase the efficacy of bacteriocin against target organisms. Harris et al.(1991) and Okereke and Montville (1991) demonstrated the synergistic effect of NaCl on bacteriocin activity. However, the antagonistic effect of sodium chloride on inhibition of spore outgrowth by nisin was reported by Bell and DeLacy (1985).

Several studies have addressed the influence of food emulsifiers on the activity of nisin. Henning et al.(1986) demonstrated an antagonistic effect of several emulsifiers (mono- or diglycerides) on the antimicrobial activity of nisin in tomato juice. However, experiments (Jung et al.,1992) with Tween 80 in half-and-half cream systems clearly showed an enhancement effect of the emulsifier on nisin activity against *Listeria monocytogenes*. Blackburn et al.(1989) also demonstrated similar nisin enhancement effects against

Streptococcus agalactiae and *Listeria monocytogenes* in milk with monoacylglycerols (lauric and oleic acids) which act as non-ionic emulsifiers.

It is now apparent that interactions of nisin with food components is a major limitation in the application of nisin in a variety of food processing and preservation technologies. In order to increase the potential applications of nisin as a natural effective food preservative in various foods, further research on understanding of the interactions between nisin and food components is necessary to optimize the efficacy of nisin in foods.

We hypothesize that nisin interacts with food lipids such as phospholipids by hydrophilic interactions between the positively charged nisin molecule and the negatively charged head group of phospholipids, resulting in a loss of nisin availability for microbial inhibition. Phospholipids are known to bind strongly with divalent cations such as calcium or magnesium ions (Ohki,1969). Therefore, as a cationic polypeptide, nisin may compete with calcium ions to bind to phospholipid or other anionic components.

In this study, we investigated the effect of Ca^{2+}, Mg^{2+} and Na^{+} on the antimicrobial activity of nisin in foods and beverages.

MATERIALS AND METHODS

Nisin and Activity Assay

High potency grade nisin was obtained from Alplin and Barrett, Ltd.

(Trowbridge, U.K.) which contained 45.5 x 10^6 IU/g (lot code #5150J). Stock solutions (50,000 IU/ml) were prepared by solubilizing in distilled water acidified to pH 2 with hydrochloric acid and kept frozen at -80°C until use. Nisin activity was determined by agar well diffusion bioassay using *Pediococcus pentosaceus* FBB-61-2 as a sensitive indicator organism as previously described (Jung et al.,1992). Estimates of nisin activity in samples were obtained by measuring inhibition zone size with a dial micrometer and comparing these with linear regression lines($r^2 \geq 0.95$) obtained by plotting the zone diameter versus $\log_{10}$ concentration of nisin.

Model Food Systems

Half-and-half cream was obtained from a local dairy. Pinot Noir (1989 harvest) red wine was produced from grapes obtained from the Oregon State University vineyard. After the primary yeast fermentation (10days at 18°C), the new wines were frozen in air-tight vessels until use. Red wine (1990) was purchased from a local market. Nisin (150 U/ml) was added into red wines with various concentrations of calcium chloride from 0% to 2%(w/v) and kept at 4°C for 1 month. The residual nisin activity in wines was determined at the beginning and the end of storage.

As a model food system, baby foods (Gerber, Second Food brand, 71g) were chosen, because they are homogeneous, sterile, and have been analyzed for composition and nutrient value (Ref.; Gerber catalog). Sample food was diluted

with 1 part of distilled H_2O and mixed homogeneously in sterilized Whirl-Pak bags (Nasco, Ft. Atkinson, WI). Homogenized food slurry with nisin and/or metal ions was centrifuged. The residual nisin activity in the supernatant of each food was determined.

Salts

Calcium chloride (MC&B, Rutherford, N.J.) and Magnesium chloride (J.T Baker Chem. Co., Philipsburg, N.J.) were used as divalent cations. Sodium chloride (VWR Scientific, N.J.) was used as a monovalent cation.

RESULTS AND DISCUSSION

Nisin retained far less activity (10.4% of original activity) in half-and-half cream as compared to those in phosphate buffer (pH 6.0) and in skim milk as shown with Table1. With 2%(w/v) of calcium chloride, nisin activity increased to 120.8% and 151.6% in the phosphate buffer and skim milk, respectively. Nisin activity in half-and-half with 2% calcium chloride had a residual activity of 46.9%. The same concentration of magnesium chloride slightly increased nisin activity in milks. On the other hand, sodium chloride at 1%(w/v) did not show an enhancement effect on nisin activity, rather than slightly diminished nisin activity in buffer and half-and-half. These results agreed with those of Bell and DeLacy (1985) who also observed an antagonistic effect of NaCl on nisin

activity. Figure 1 shows the effect of concentration of calcium chloride in half-and-half on nisin activity. As concentrations of calcium chloride increased, nisin activity in half-and-half significantly increased.

Daeschel et al.(1991) observed nisin activity decreased with time in red wines. In this study, nisin was added into red wines with various concentrations of calcium chloride to determine the enhancement effect of calcium ions on nisin activity in red wines. After one month storage of samples at 4°C, the residual nisin activity in red wines decreased to 23.2%. However, calcium chloride containing wines retained nisin activity much more than the control, depending on the concentrations of calcium ions. As concentration of calcium chloride increased, greater nisin activity was detected in red wine (Fig. 2). It was proposed that the binding of the polymerized tannins in aged red wine to nisin molecules caused nisin activity lost in red wines (Bower, at al.,1992). We assume that it is probably due to the interaction between the hydroxyl groups of the polymerized tannin and nisin molecule. Therefore the possible binding of calcium chloride to the pigment may account for the observation of nisin activity in red wines.

Several observations of poor preservation effect of nisin in meat systems have been reported in the literature. Experiments with model foods (Gerber baby foods) such as beef (5.63% fat), beef & carrot mixture (2.65% fat), ham (8.45% fat), chicken (8.45% fat), broccoli & chicken mixture (1.77% fat), and turkey (7.04% fat) clearly showed that nisin activity significantly decreased in

high fat containing foods as compared to low fat foods. However, nisin with divalent cations such as calcium or magnesium ions, retained much higher activity in these foods as illustrated on Figure 3. The size of inhibition zone was dependent upon the concentration of active nisin in each sample. Calcium chloride represented in lane 1 increased nisin activity in every food tested, compared to those of controls shown in lane 3. Unlike in half-and-half cream, magnesium chloride (lane 2) also showed an enhancement effect in high fat containing foods. The enhancement effect of magnesium ions on nisin activity in food systems was little bit less than that of calcium ions. Figure 4 illustrates the enhancement effect of calcium chloride on antimicrobial activity of nisin at various concentrations in chicken (8.45% fat) and peaches (0% fat). Nisin activity in peaches was almost the same as that of the control which was in water pH adjusted to that of peaches. Once again, chicken retained far less nisin activity than control. When chicken contains calcium chloride, residual nisin activity in each sample was almost the same as that of control.

In previous experiments, we had observed that phospholipids have an antagonistic effect against nisin. To test the enhancement effect of monovalent and divalent cations on nisin activity reduced by phospholipid, phosphatidyl choline was mixed with nisin in phosphate buffer (pH 6.0) and then calcium chloride, magnesium chloride or sodium chloride was added into the mixture. The results (Table 2) evidently showed that calcium chloride had a significant effect, but magnesium chloride and sodium chloride did not show enhancement

effects on the reduced nisin activity by phosphatidyl choline in buffer solution (pH 6.0). We believe that divalent cations can enhance or restore nisin activity in certain foods which contain nisin interfering components like phospholipid or other anionic components. However, the interaction between phospholipid and divalent cations could be related to the degree of the dissociation of the polar groups of phospholipid, and the binding of divalent cations varies with different phospholipid. Ohki (1969) observed that calcium ion chelated with phosphatidyl choline molecules and more strongly with phosphatidyl serine molecules, but the binding of magnesium ions was not as strong as that of calcium ions with the negatively charged sites of phospholipid head groups. We also observed that the enhancement effect of magnesium ions on nisin activity in food systems was intermediate between that of sodium ions and calcium ions. These results agree with the observations of Ohki(1969) that the degree of binding of magnesium ions with the phospholipid lies between that of sodium ions and calcium ions. Phosphatidyl serine which has one more negative charge, however, bound with both calcium and magnesium ions at pH range of above pH 4. These observations may explain our results which magnesium chloride did not show enhancement effect in buffer solution (pH 6.0) with phosphatidyl choline, while calcium chloride had an enhancement effect. However, magnesium chloride did increased nisin activity significantly in the meat and poultry food systems. We believe that since meats and poultry have a greater variety of phospholipids, which can interact more strongly with divalent cations, hence binding with nisin

molecules is prevented. Therefore, certain divalent cations can enhance the antimicrobial efficacy of nisin in certain foods.

It is interesting that the efficacy of nisin can be controlled by different cations and their concentrations in food systems.

Table 1. Effect of metal ions on nisin activity in phosphate buffer (pH 6.0), skim milk and half-and-half cream.

Samples	Nisin activity (%)	Samples	Nisin activity (%)
N / buffer	100	N / buffer + NaCl	89.3
N / buffer + $CaCl_2$	120.8	N / buffer + $MgCl_2$	100
N / h+h cream	10.4	N / skim milk	89.3
N / h+h + $CaCl_2$	46.9	N / skim +$CaCl_2$	151.6
N / h+h + NaCl	9.2	N / skim + NaCl	100
N / h+h + $MgCl_2$	13.5	N / skim + $MgCl_2$	112.1
N / h+h + T-80	96.3	N / skim +T-80	103.8

N: Nisin 150 IU/ml

h+h: half-and-half cream

T-80: Tween 80

Table 2. Effect of metal ions on nisin activity in phosphate buffer (pH 6.0) with phosphatidyl choline.

Samples	zone dia.(mm)	nisin activity (%)
N / buffer (pH6.0)	17.8	100
N / buffer + PC	15.5	50.7
PC / buffer	0	0
N /buffer + PC + $CaCl_2$	16.8	74.4
N / buffer + PC + NaCl	15.5	50.7
N / buffer + PC + $MgCl_2$	15.5	50.7

N: Nisin 150 IU/ml

PC: Phosphatidyl choline

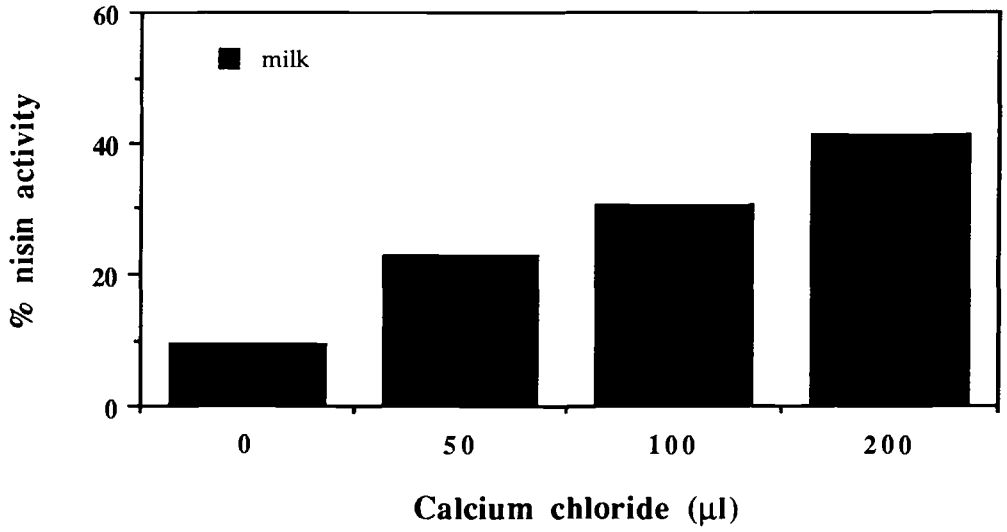


Fig. 1. Enhancement effect of calcium ions on the efficacy of nisin in milk.

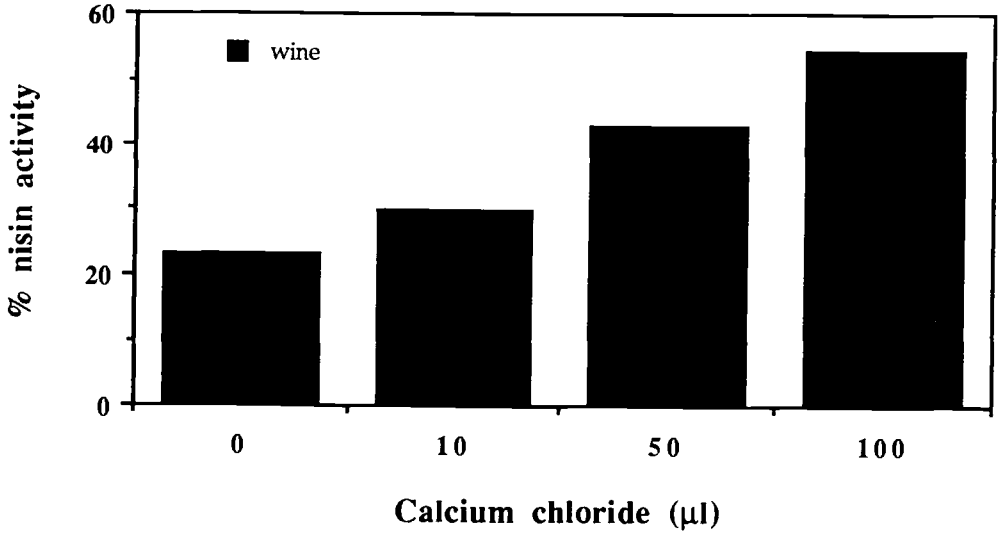


Fig. 2. Enhancement effect of calcium ions on the efficacy of nisin in red wine.

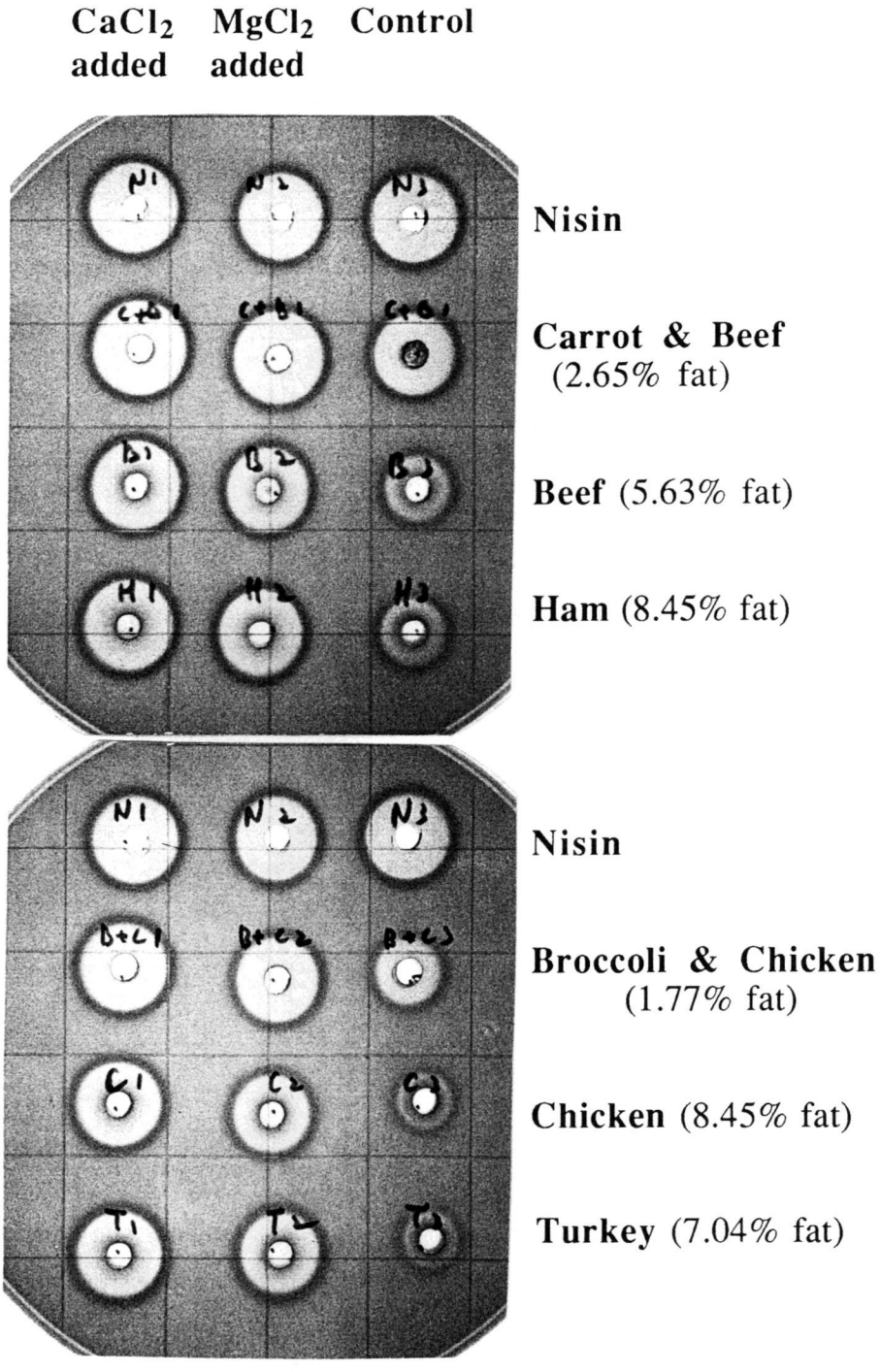


Fig. 3. Enhancement effect of calcium and magnesium ions on nisin activity in food systems.

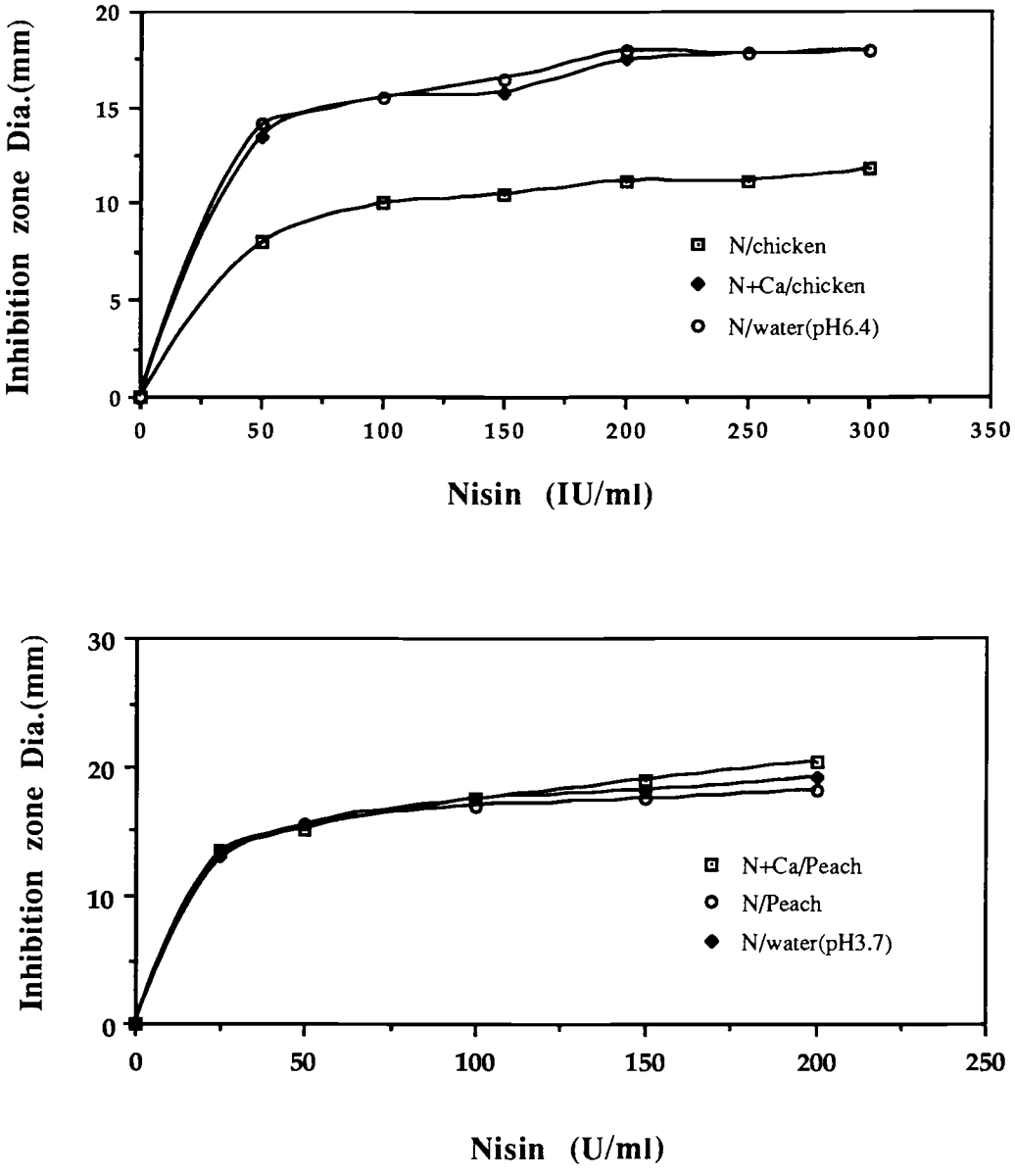


Fig. 4. Enhancement effect of calcium ions on nisin activity in high fat containing food (top) and non fat food (bottom).

Chapter 5

Some Characteristics of Nisin Related to Its Mode of Action

ABSTRACT

The effect of pH on nisin activity during storage was measured. Nisin in acetate and phosphate buffer solutions retained less activity at higher pH after 10 weeks storage at 4°C. Nisin-sensitive and resistant cells of *Leuconostoc oenos* were exposed to nisin to measure binding of nisin to the cells. Nisin was completely adsorbed to the nisin-sensitive cells, but adsorbed less to the nisin-resistant cells. The growth of *Pediococcus pentosaceus* in the presence of metal ions and nisin, was measured to test the effect of mono- and divalent cations on the antimicrobial activity of nisin. *Pediococcus pentosaceus* was completely inhibited by 20 IU/ml of nisin in MRS broth. However, in the presence of divalent cations such as Ca^{2+} or Mg^{2+}, nisin lost its inhibitory activity against *P. pentosaceus*. This bacterium in MRS media with 20 IU/ml of nisin and 1 mg/ml of calcium chloride, reached the stationary phase after a longer lag phase than the control treatment. On the other hand, Na^{+}, did not exhibit any antagonistic effect against nisin. Nisin inhibited the growth of *P. pentosaceus* in the presence of sodium chloride at concentrations of 0 to 2.34 mg/ml. We assume that exogenous divalent cations may compete with cationic nisin for binding to the membrane phospholipids which is believed to be the primary target for nisin adsorption and action.

INTRODUCTION

The mechanism of action of nisin at the molecular level against vegetative cells and spores of sensitive Gram-positive bacteria is not understood definitively.

Ramseier (1960) suggested that nisin behaves as a surface active cationic detergent and adsorption to cells appears to be the first step required for its action. Recent studies on the mode of action of nisin (Ruhr and Sahl,1985; Henning et al.,1986; Kordel and Sahl, 1986) indicated that the cytoplasmic membrane of bacteria is the major target which is disrupted by interaction of nisin with phospholipid components of bacteria.

It is known that divalent cations bind to phospholipid. The affinity of phospholipids for the different divalent cations is related to the stability of phospholipid bilayers (Ohki,1969). It appears that Ca^{2+} and Mg^{2+} are important in stabilizing the surface structures of bacteria (Repaske, 1958). Following the incorporation of these ions into the cell envelope it becomes less permeable to aminoglycosides and other compounds (Zimelis & Jackson, 1973). In addition, the cations may compete with antibiotics such as gentamicin for binding on or in the cell (Ramirez-Ronda et al.,1975).

In previous experiments, we observed that antimicrobial activity of nisin is significantly reduced in certain foods such as high fat containing foods. However, greater nisin activity remained when divalent cations were added into

high fat containing foods, probably due to the interaction of certain food components such as phospholipid with cationic metal ions instead of nisin molecules. Therefore we believe that there may be competition between cationic polypeptide nisin and divalent cations to bind with phospholipid components of bacterial membrane, resulting in change of membrane permeability.

This study was designed to confirm the adsorption of nisin to bacterial cells and to determine the enfluence of mono- and divalent cations on the antimicrobial activity of nisin against gram positive bacteria which are sensitive to nisin.

MATERIALS AND METHODS

Microorganisms

Leuconostoc oenos was grown in WLAB medium (Daeschel et al., 1991) and incubated at 30 °C for cultivation. *Pediococcus pentosaceus* FBB-61-2 (Daeschel and Klaenhammer, 1985) was cultured in MRS broth (Difco, Detroit, MI) and incubated at 37 °C for growth.

Nisin and Nisin Activity Assay

A nisin preparation was obtained from Aplin and Barret Ltd. (Trowbridge, U.K.) that contained 45.5×10^6 IU /g (lot code #5150J). A working stock solution of 50,000 Units /ml was prepared by solubilizing the nisin

preparation in 0.02 N HCl (~pH 2.0) and storing at 4 °C in the dark. Nisin activity was determined by bioassay using *Pediococcus pentosaceus* FBB-61-2 as the sensitive indicator strain. Estimates of nisin activity in samples were obtained by measuring inhibition zone size with a dial micrometer and comparing these with linear regression lines($r \geq 0.99$) obtained by plotting the zone diameter versus $\log_{10}$ concentration of nisin.

pH Effect

Citrate-phosphate buffer and acetate buffer were prepared at different pH values from pH 2.0 to 7.0. Nisin(100 IU/ml) was added to the buffers and kept at 4°C for 10 weeks at which time residual nisin activity was measured.

Adsorption and Desorption of Nisin

Nisin sensitive and resistant cells of *Leuconostoc oenos* Ey2d grown in WLAB medium were centrifuged and washed twice with citrate-phosphate buffer (pH 6.5) and resuspended with same buffer (pH 6.5) to obtain various cell concentrations (optical density 0, 0.5, 1.0, and 1.5 at 600 nm). Nisin at levels of 50, 100 and 150 IU/ml was then added to the cells. After exposure to nisin for 30 minutes at room temperature, cells were spun down by centrifugation and residual nisin activity in the supernatant was measured. The difference in the amount of nisin activity between control and sample cells was considered as nisin bound to the cells. Cells collected by centrifugation were washed twice with

buffer and 0.1% of NaCl added and vortexed vigorously to desorb nisin from cells. Sodium chloride treated samples were centrifuged again to remove cells and the residual nisin activity was measured in the supernatant, which was considered as desorbed nisin from cells.

The Effect of Mono- and Divalent Cations

Pediococcus pentosaceus FBB-61.2 was inoculated at a 0.2% (v/v) of a standardized overnight culture into 5 ml aliquots of MRS broth (10 x 100 mm tubes). Sodium chloride, calcium chloride or magnesium chloride at various concentrations were added to tubes containing 20 IU/ml of nisin or no nisin. Bacteria were incubated at 37°C, and bacterial growth was monitored by measuring optical density of the cells at 600 nm, using a Spectronic 20 (Milton Roy Company) for 24 hrs.

RESULTS AND DISCUSSION

pH Effect on Nisin Activity

It is known that nisin is stable at pH 2.0 indefinitely, but becomes inactivated at above neutral pH.

To measure the effect of pH on nisin activity during storage, nisin (100 IU/ml) was added to acetate and phosphate buffers at various pH levels, and then kept at 4°C for 10 weeks. The biological activity of nisin did not change

significantly in the buffers until about 3 weeks of storage. It was observed that nisin activity decreased at higher pH with more than 3 weeks storage. Nisin activity remained at 100% with pH 2.0 and at 36.3% with pH 7.0 in citrate-phosphate buffer, and 90.4% with pH 3.0 and 49.2% with pH 6.0 in acetate buffer after 10 weeks at 4°C (Fig. 1).

Adsorption of Nisin to the Bacterial Cells

Ramseier(1960) reported that nisin was strongly adsorbed by nisin sensitive cells of *Cl. butyricum*, but not adsorbed by cells which had been made resistant.

However, our results with *Leuconostoc oenos* indicated that nisin was adsorbed to the nisin sensitive and resistant cells of *Leuconostoc oenos* Ey2d at pH 6.5. With both types of cells, as concentrations of cells increased, the recovered nisin activity from cells decreased as illustrated in Figure 2. Nisin at concentrations of 100 and 150 IU/ml was completely adsorbed to preparations of high nisin sensitive cell densities (A_{600} = 1.5), but less adsorbed to the nisin resistant cells at the same densities. Nisin was desorbed from nisin-resistant cells, but not from sensitive cells (Data not shown). These results suggest that nisin may penetrate into cytoplasmic membrane after initial adsorption to the cell surface of sensitive cells. However, nisin may not cross into the cytoplasmic membrane of nisin-resistant cells and may be desorbed from the cells under certain conditions. We assume that nisin resistance is developed by alteration of

cellular fatty acid composition to saturated fatty acids. In previous experiments, it was observed that phospholipids and fatty acids interact with nisin. We believe that interaction of nisin with cellular lipids renders celluar structure unstable, resulting in changing permeability. Juneja and Davidson (1992) observed a correlation between lipid composition and susceptibility of *L. monocytogenes* to antimicrobials. They demonstrated that *L.monocytogenes* grown in the presence of saturated fatty acids increased resistance to several antimicrobials. On the other hand, unsaturated fatty acid lowered resistance of *L. monocytogenes* to the same antimicrobials.

The Effect of Metal Ions on the Antimicrobial Activity of Nisin

Pediococcus pentosaceus FBB-61.2 which is sensitive to nisin was exposed to nisin in the presence of different concentrations of sodium chloride, calcium chloride, or magnesium chloride in the culture media to determine the effect of mono- and divalent cations on the antimicrobial activity of nisin.

The growth of *Pediococcus pentosaceus* FBB-61.2 was completely inhibited by 20 IU/ml of nisin in the MRS broth. However, divalent cations such as Ca^{2+} and Mg^{2+} counteracted the inhibitory activity of nisin in MRS media. Figure 3 illustrated the results of the effect of calcium and magnesium ions on the inhibitory activity of nisin against *Pediococcus*. Nisin was inactive against *Pediococcus* in MRS media when calcium chloride or magnesium chloride was added, with increasing concentrations decreasing nisin activity. With calcium

chloride at concentration of 1 mg/ml, the growth of cells in the presence of nisin reached maximum stationary phase. Calcium chloride (< 0.2 mg/ml) did not exhibit any counteractive effect against nisin activity. *P. pentosaceus* also survived in the presence of nisin with much higher concentrations of magnesium ions than calcium ions (Fig. 3).

However, sodium chloride did not give any antagonistic effect against nisin. Nisin completely inhibited the growth of *P. pentosaceus* in the presence of sodium chloride at the concentrations of 0 to 2.34 mg/ml. Sodium chloride alone at the concentration of 2.34 mg/ml did not give any inhibitory effect against *Pediococcus* (Fig. 4).

The results of this study indicate that Ca^{2+} and Mg^{2+} may compete with nisin for binding with membrane phospholipids which are known to be the primary target for nisin action. Therefore, interaction of divalent cations with membrane phospholipids may prevent nisin adsorption to cell membranes.

D'Amato et al.(1975) also reported that calcium and magnesium ions increased minimun inhibitory concentration (MIC) of several antibiotics against *Pseudomonas*. Ramirez-Ronda et al.(1975) suggested that the enhanced resistance of *P. aeruginosa* in the presence of Ca^{2+} and Mg^{2+}, is associated with decreased uptake of gentamicin by the bacteria. However, the mechanism by which divalent cations exerted this effect was not fully elucidated.

We assume that nisin exhibits its antimicrobial action by interaction with negatively charged phospholipids of the bacterial membrane and these

interactions can be blocked by divalent cations. The interaction of phospholipids with Ca^{2+} has been studied by the use of binding of Ca^{2+} on monolayers (Rojas and Tobias,1965; Kimizuka et al.,1967; Papahadjopoulos, 1968). The interaction between phospholipids and metal ions (Na^{+}, Mg^{2+}, Ca^{2+}) could be related to the degree of the dissociation of the polar groups of phospholipids, and the binding of divalent metals may vary with different phospholpids. Ohki (1969) observed that with the phosphatidyl serine (PS) molecule, calcium binding is much stronger than with phosphatidyl choline (PC) molecules, and for magnesium ions, the binding is not as strong as the binding of calcium ions with the negative sites of phospholipid head groups. Ohki (1969) also demonstrated that the degree of binding of magnesium ions with the phospholipid lies between that of sodium ions and calcium ions. The effect of the membrane resistance due to the binding of magnesium metal ion with the PS molecule is only 1/5 - 1/10 times that of calcium ions. Thus, the membrane becomes more stable and more rigid in the presence of calcium ions. These observations may explain why calcium ions were more effective in counteracting nisin activity than magnesium ions.

Most membranes contain considerable amounts of phospholipids, including PS, upon which calcium ions interact strongly. This may also explain why nisin does not inhibit Gram-negative bacteria. In Gram-negative bacteria, none of the phospholipids of the outer membranes are exposed (Fig. 5; Hancock,1991), which could make nisin unreactive to the cell membrane. However, in a study by Nikaido and Vaara (1985), the outer membrane of "deep

rough" lipopolysaccharide (LPS) mutants was shown to contain much larger amounts of phospholipids, some of which were apparently located in the outer leaflet. Stevens et al.(1992) observed that "deep rough mutant" cells of *Salmonella typhimurium* were sensitive to 50 μg/ml of nisin, while parent cells were resistant, and that EDTA treated Gram-negative cells become sensitive to nisin, presumably by disruption of the outer membrane.

Therefore, we believe that the alteration of the outer membrane of Gram-negative bacteria, such as eliminating cationic metals by treatment with chelating agents, changes membrane permeability and thus provides nisin access to the cytoplasmic membrane. This then results in nisin-mediated inhibition as in Gram-positive bacteria. On the other hand, providing exogenous divalent cations to the Gram-positive cells increases membrane stability and hence makes membrane phospholipids unaccessible to nisin action by blocking the negative charge of the phospholipids in the membrane.

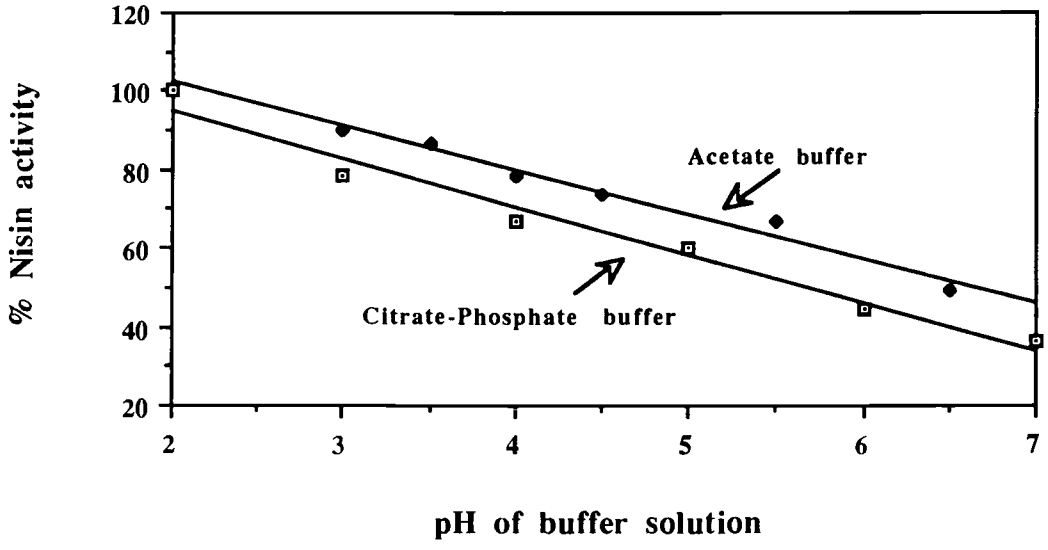


Fig. 1. Effect of pH on nisin activity during storage in buffer solutions.

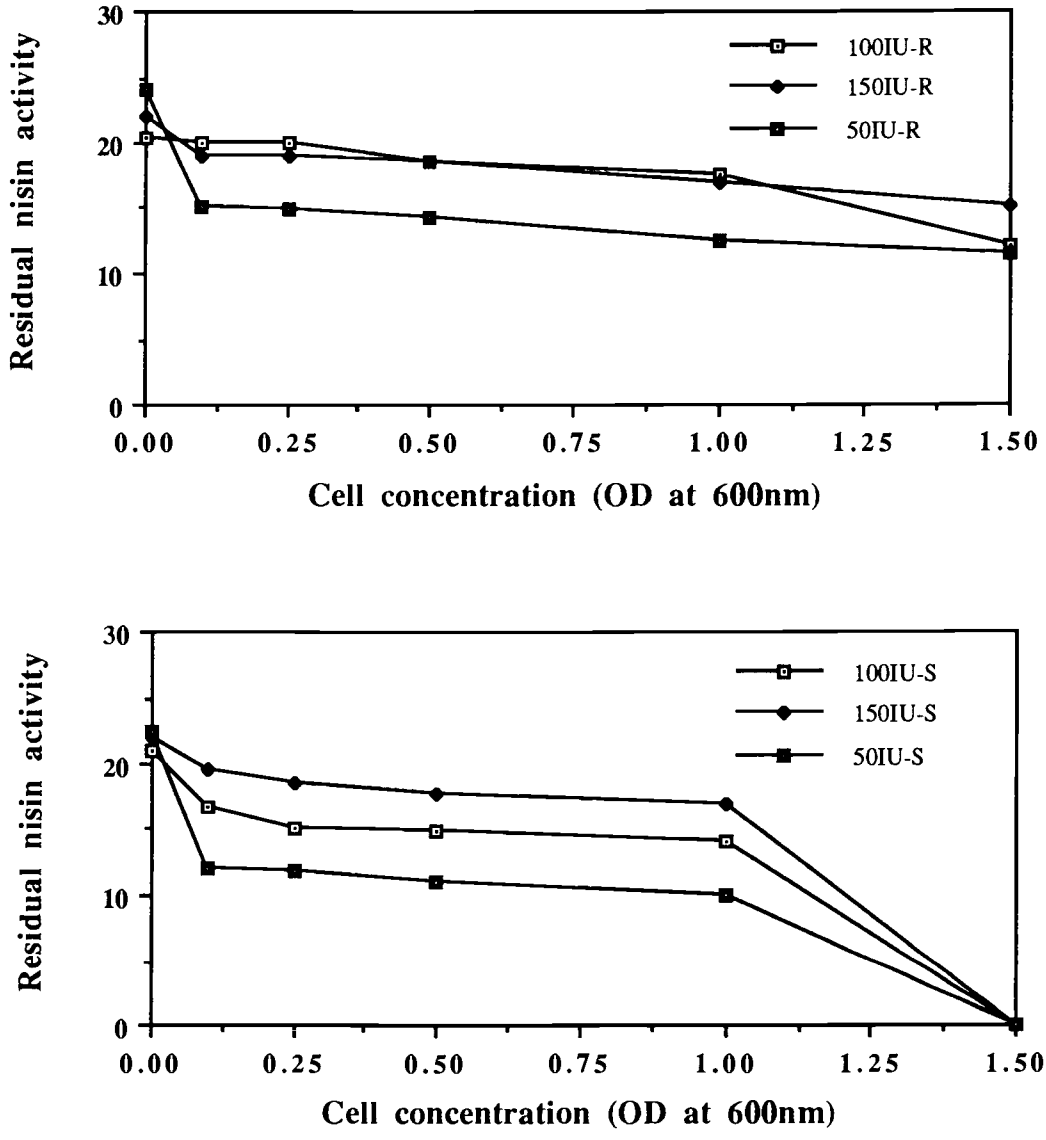


Fig. 2. Adsorption of nisin onto nisin-sensitive cells (top) and nisin-resistant cells (bottom) of *L. oenos* Ey2d.

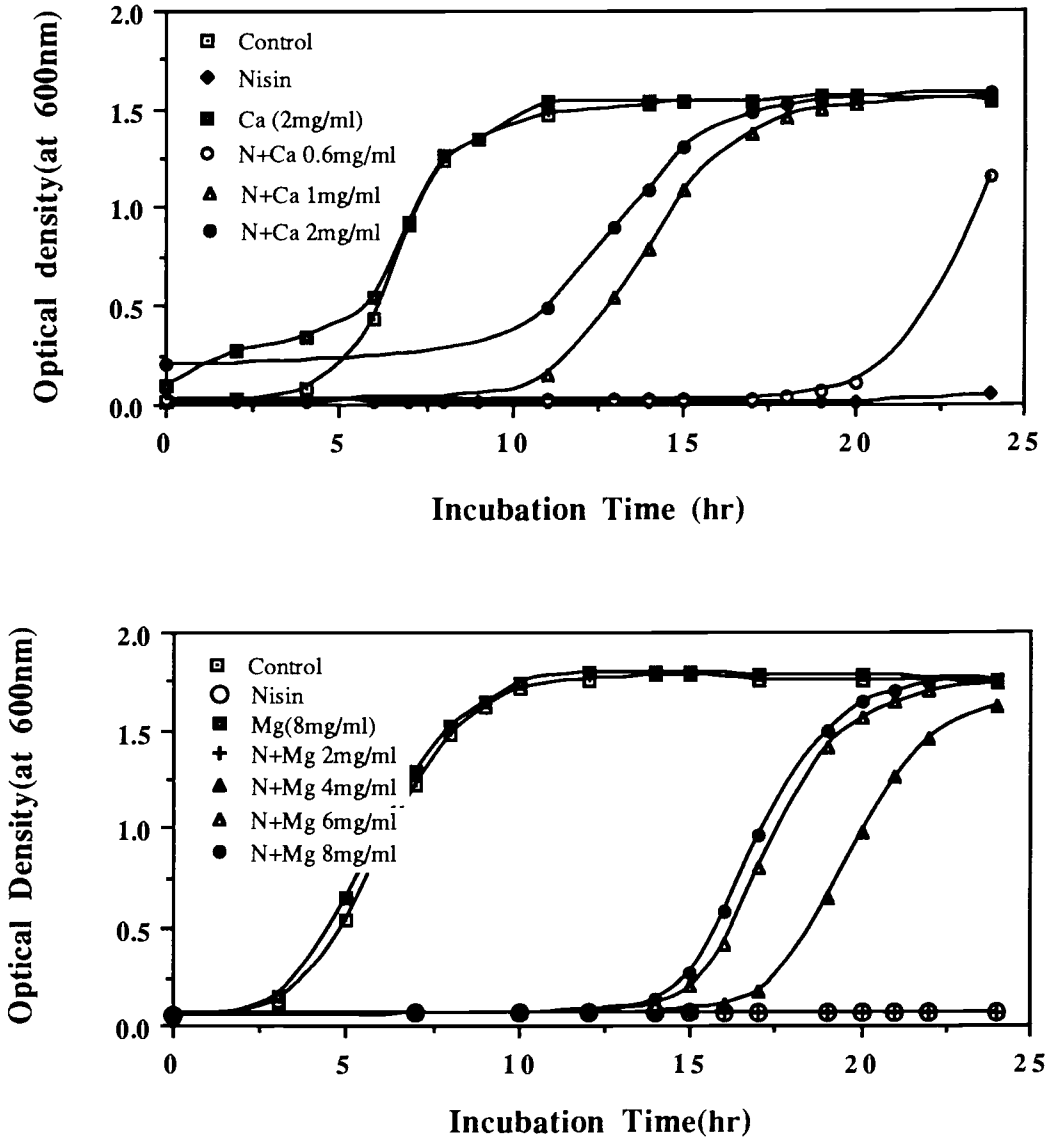


Fig. 3. Effect of calcium ions (top) and magnesium ions (bottom) on nisin activity against *Pediococcus pentosaceus* FBB-61-2.

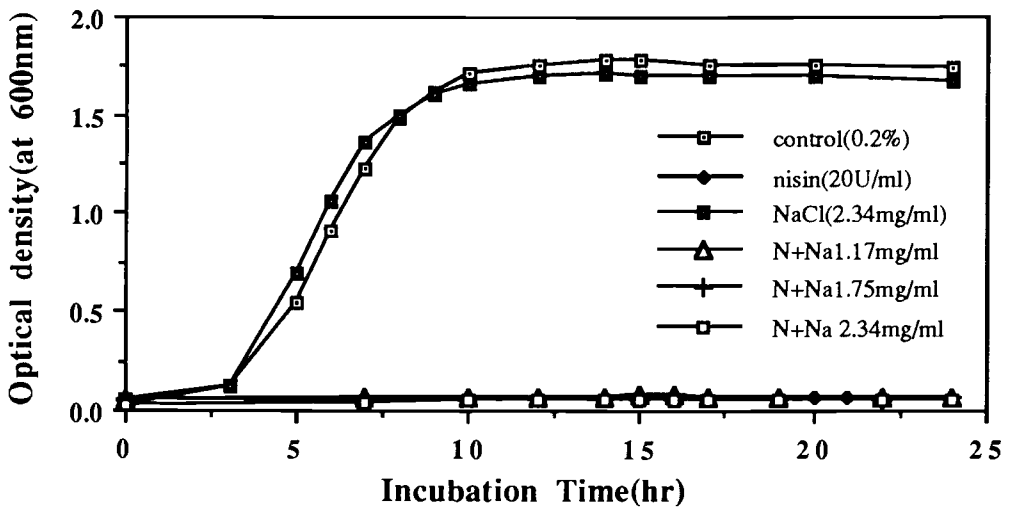
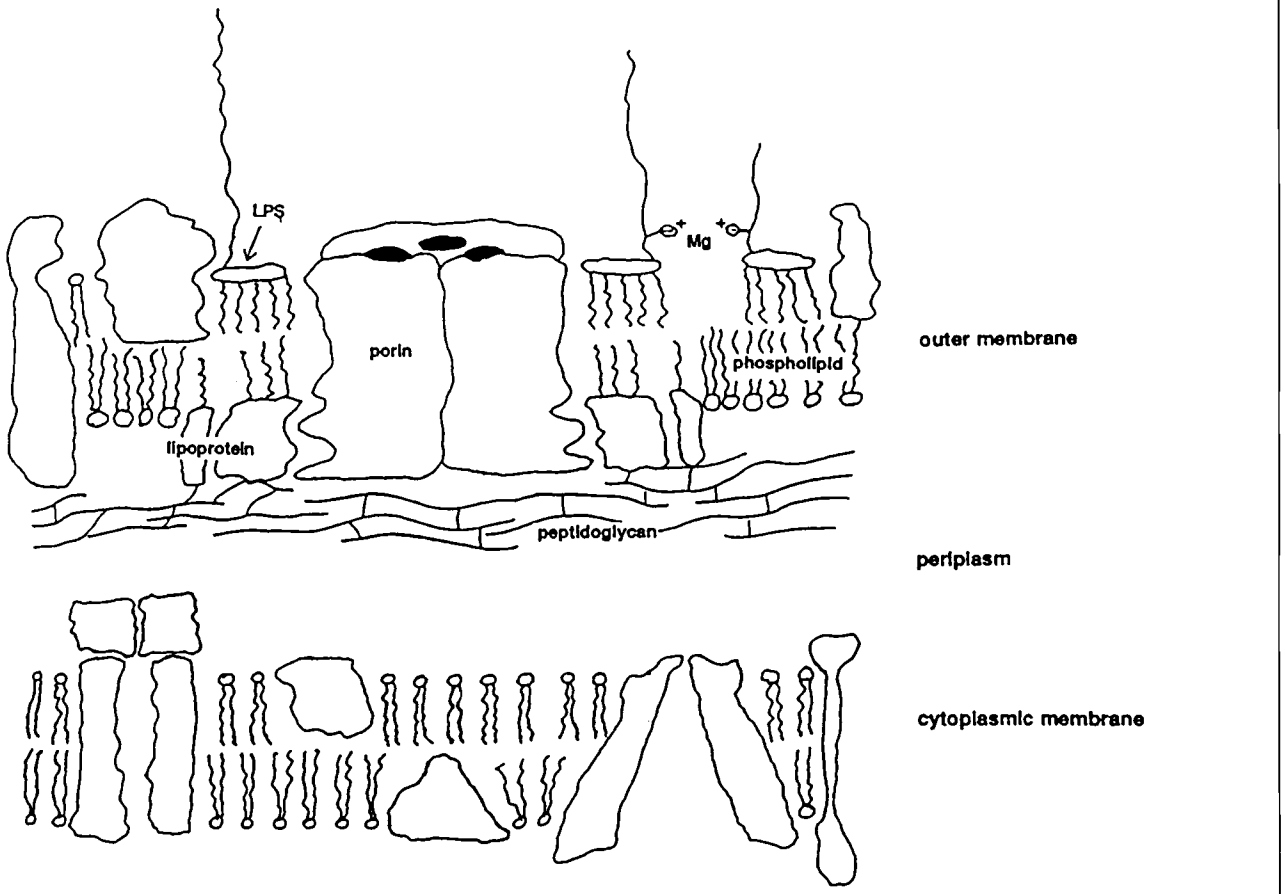


Fig. 4. Effect of sodium ions on nisin activity against *Pediococcus pentosaceus* FBB-61-2.



Note the asymmetric distribution of lipid-containing species in the outer membrane, with a paucity of phospholipids in the outer leaflet. The outer membrane is associated through strong noncovalent or occasionally covalent interactions of proteins with the underlying peptidoglycan.

Fig. 5. Major features of the cell envelope of a Gram-negative bacterium (from Hancock, 1991).

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Appendices

1. Labeling of nisin with fluorescein isothiocyanate

2. Nisin's legal status in the world

APPENDIX 1. LABELING OF NISIN WITH FLUORESCEIN ISOTHIOCYANATE (FITC)

OBJECTIVE

Nisin contains no aromatic amino acids, thus it has no absorbance at 280 nm. However, it has three lysine residues which can be labeled with certain fluorescent chemicals such as fluorescein isothiocyanate, under certain conditions. Fluorescein isothiocyanate (FITC) labeled nisin could be visuallized through fluorescence microscopy and measured quantitatively by fluorescence spectrophotometer. Nisin activity is usually measured by agar well diffusion bioassay, but it takes two days to detect and its activity may be affected by many factors. HPLC methods also can be applied, but only large amounts(>1000 IU) of nisin is detected. Similar limitations exist with protein electrophoresis methodology. If FITC labeled nisin retains its antimicrobial activity, estimation of residual nisin in food systems is more convenient and fast than other methods. It also could be useful tool for understanding of the mechanism of nisin action by visualizing target locations.

EXPERIMENTAL DESIGN AND METHODS

A stock solution of FITC was made by dissolving 2 mg FITC in 5 ml of NaOH-glycine buffer (pH 9.0). Nisin 500µl of 50,000 IU/ml was mixed with 4.5 ml FITC stock solution and then stand for 1 hr in a dark container at room temperature, occasionally shaking. Controls were consisted of nisin itself without FITC and FITC without nisin. Unreacted (unbound) FITC was removed by dialysis in acetate buffer (pH 4.0) with a spectrapore membrane (MW cut off 2,000) for 24 hrs at 4°C, changing the buffer every 4hrs.

Reaction mixtures were analyzed by HPLC(High-performance liquid chromatography). Fractions were collected and measured nisin activity by agar well diffusion bioassay using sensitive indicator orgarnism.

Analytical reversed-phase HPLC separations were performed on the Water system: two Solvent Delivery Module 110B, autoinjector, gradient programmer(controller) 421A. For detection, ultraviolet detector with variable wavelength(Beckman 163) combined with integrator 427(Beckman). Zorbax protein plus column(4.6mm x 250mm) was used as stationary phase. Elution was with a linear gradient from 25% to 55% of acetonitrile/0.1% trifluoroacetic acid in water in 30min, using a flow rate of 1.5 ml/min and monitoring the effluent at 222 nm.

Reaction mixture (called "FITC labeled nisin") was exposed to milk fat and to nisin-resistant and sensitive cells of *Leuconostoc oenos* Ey2d, and then

was viduallized under Fluorescence microscopy.

Fluorescence microscopy was performed using a Reichert One-Ten Microscope with Vertical & Transmitted Light Fluorescence System (AO Reichert Scientific Instruments, NY).

RESULTS AND DISCUSSION

HPLC analysis of a labeled nisin revealed the presence of a number of "slow-eluting" components (Fig. 1). The major components were analyzed for nisin activity. Nisin was labeled by FITC and retained its activity. Among "slow-eluting" peaks, three peaks (retention time of 17.52, 18.08 and 18.72) showed nisin activity, suggesting polymerization and/or labeling of nisin with FITC occur. However, the rest of peaks did not exhibit nisin activity. It was also observed that free FITC peaks were appeared at the retention time of 7.2 min. and 8.07 min., probably due to aggregation of FITC in the low pH at which it was dialyzed.

Milk fat containing FITC labeled nisin was viduallized under fluorescence microscope. It was observed that FITC labeled nisin was adsorbed to milk fat globule, specifically, outside rim of the globules.

FITC- labeled nisin also adsorbed to both nisin-resistant and sensitive cells of *L. oenos*. Nisin-resistant cells were able to grow after exposed to FITC-labeled nisin, but nisin sensitive cells could not survive at the same conditions.

The major problem in labeling nisin with FITC was the detection and the complete removal of the unbound FITC from the labeled nisin mixture.

Nisin lose activity at high pH, but the procedure necessitated treating nisin at high pH (above pH 8.0) for labeling with FITC since FITC reacts with undissociated amine groups. Thus, after interaction of nisin with FITC for 1 hr at pH 9.0, the reaction mixture was adjusted to pH4.0 for removal of free FITC. However, solidification of free FITC was observed at low pH. Therefore, the optimization of labeling conditions is needed for better results.

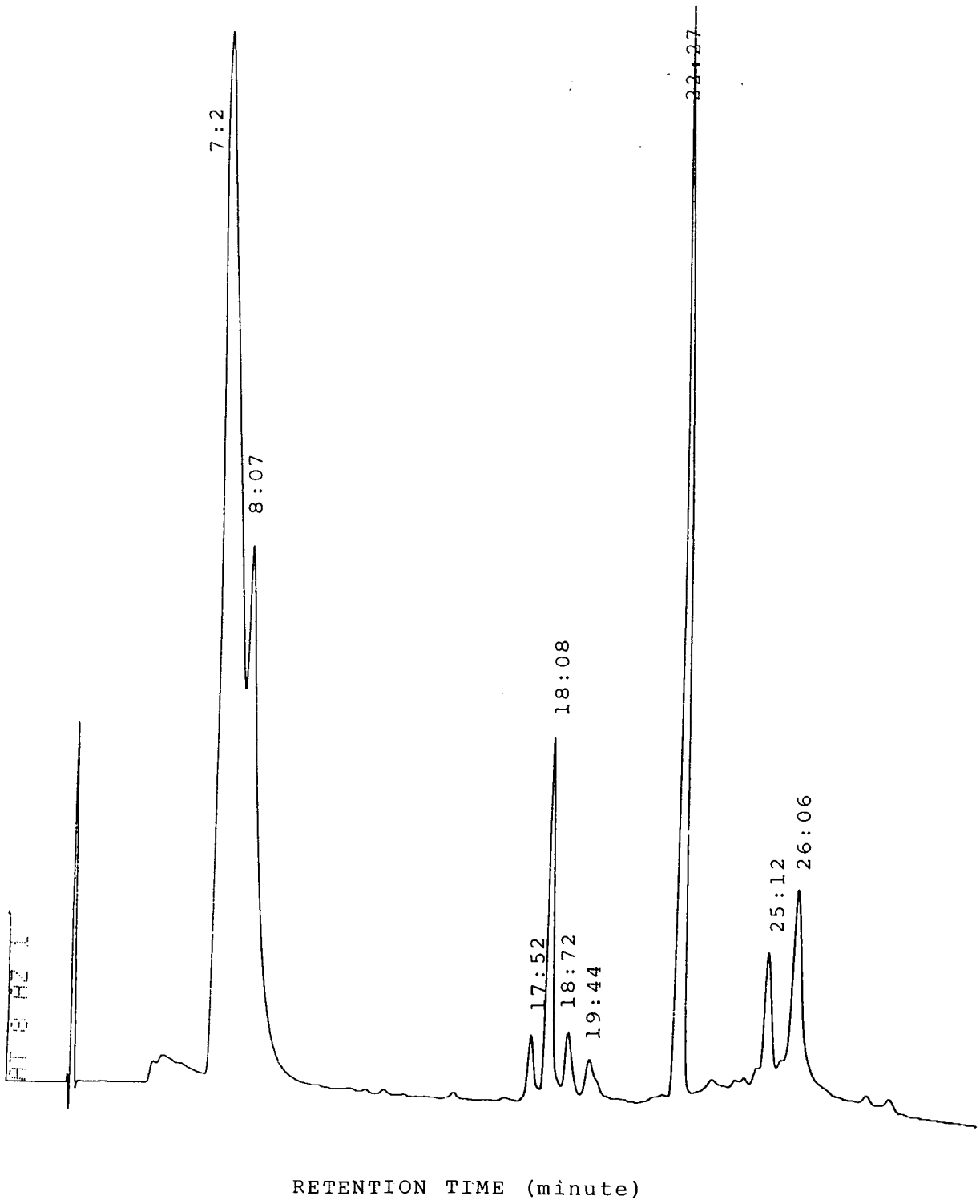

Fig. 1. Chromatogram (HPLC, 222 nm) of fluorescein isothiocyanate (FITC) labeled nisin.

APPENDIX 2. NISIN'S LEGAL STATUS IN THE WORLD
[cited from Delves-Broughton (1990) and Ray (1992)]

Country	**Food in which nisin is permitted**	**Maximum level of addition** (IU nisin/g food product)
Abu Dhabi	Pasteurized milk Flavored milk Long-life milk Processed cheese Cheese Other dairy products Canned vegetables	No limit
Argentina	Processed cheese	500
Australia	Cheese Canned tomatoes Canned tomato puree & paste Canned soups	No limit
Bahrain	Pasteurized milk Flavored milk Long-life milk Processed cheese Other dairy products Canned vegetables	No limit
Belgium	Cheese	100
Bolivia	Use not prohibited in foods	No limit
Brazil	Pasteurized cheese Processed cheese Requeijao	500
Bulgaria	Cheese Ice for storing fresh fish	200
Chile	Food products	4,000
Colombia	Processed cheese	500
Costa Rica	Cheese products	No limit

Cyprus	Cheese Clotted cream Canned vegetables Bakery products & fillings Mayonnaise	No limit
Czechoslovakia	Processed cheese Prepared foods Semi prepared foods Canned vegeatbles Baby foods-dairy & vegetabe	500
Denmark	Canned processed cheese	500
Dubai	Cheese Pasteurized milk Flavored milk Long-life milk Other dairy products Canned vegetables	No limit
Egypt	Processed cheese	500
Eire	Processed cheese	500
Finland	Processed cheese	480
France	Processed cheese	No limit
Gibraltar	Same as U.K.	No limit
Hong Kong	Same as U.K.	No limit
India	Cheese Processed cheese	1,000
Italy	Cheese Canned vegetables Confectionary creams	500 100
Jordan	Processed cheese	500
Kuwait	Processed cheese	4,000
Malaysia	Canned foods & cheese	No limit
Malta	Same as U.K.	No limit
Mexico	Food products	No limit

Netherlands	Factory cheese Quarg Certain processed cheese Cheese powder	500 800
New Zealand	Processed cheese	500
Peru	Nisin is permitted additive	No limit
Philippines	Processed cheese	4,000
Poland	Ripened natural cheese Processed cheese	4,000
Portugal	Processed cheese	500
Qatar	Milk Milk products	No limit
Saudi Arabia	Some foods & dairy products	No limit
Singapore	Cheese Canned foods	No limit
South Africa	Processed cheese products Some other cheeses	No limit
Spain	Processed cheese	500
Sweden	Cheese & margarine Cheese with fresh meat Processed cheese	500
Taiwan	Cheese	1,000
Thailand	Processed cheese	4,000
Trinidad	Canned foods Cheese Clotted cream	No limit
Turkey	Various cheeses	4,000
United Kingdom	Cheese Canned foods Clotted cream	No limit
United States of America	Pasteurized processed cheese spread	10,000
Uruguay	Processed cheese	4,000

Russia (former USSR)	Dietetic processed cheese	8,000
	Canned vegetable products	4,000