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Applications of the bacteriocin, nisin

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Abstract

Nisin was first introduced commercially as a food preservative in the UK approximately 30 years ago. First established use was as a preservative in processed cheese products and since then numerous other applications in foods and beverages have been identified. It is currently recognised as a safe food preservative in approximately 50 countries. The established uses of nisin as a preservative in processed cheese, various pasteurised dairy products, and canned vegetables will be briefly reviewed. More recent applications of nisin include its use as a preservative in high moisture, hot baked flour products (crumpets) and pasteurised liquid egg. Renewed interest is evident in the use of nisin in natural cheese production. Considerable research has been carried out on the antilisterial properties of nisin in foods and a number of applications have been proposed. Uses of nisin to control spoilage lactic acid bacteria have been identified in beer, wine, alcohol production and low pH foods such as salad dressings. Further developments of nisin are likely to include synergistic action of nisin with chelators and other bacteriocins, and its use as an adjunct in novel food processing technology such as higher pressure sterilisation and electroporation. Production of highly purified nisin preparations and enhancement by chelators has led to interest in the use of nisin for human ulcer therapy, and mastitis control in cattle.

Introduction

Nisin is a low molecular weight antimicrobial protein or bacteriocin produced by certain strains of *Lactococcus lactis* subsp. *lactis* (hereafter referred to as *L. lactis*). It has been used as a food preservative for over 30 years. Recent developments in the production of pure nisin preparations have resulted in its re-evaluation for veterinary and pharmaceutical uses.

Nisin was first discovered in the late 1920s and early 1930s when it was described as a toxic substance present in milk which adversely affected the performance of cheese starter cultures (Rogers & Whittier 1928; Whitehead 1933). Notable landmarks in its history and development as a food preservative are its characterisation at the National Institute for Research in Dairying, Shinfield, Reading in the United Kingdom in 1947 (Mattick & Hirsch 1947), the first investigation into its potential as a food preservative by Hirsch et al. in 1951 and research on its use as a preservative in processed cheese in 1952 (McClintock et al. 1952). The commercial development of the nisin preparation called Nisaplin was carried out by Aplin & Barrett in 1957. Nisin was shown to be non-toxic by various workers in 1962 (Frazer et al. 1962; Hara et al. 1962). The molecular structure was elucidated in 1971 (Gross & Morell 1971). Commercial nisin concentrates were defined in terms of identity and purity by JECFA in 1969 (WHO 1969) and the creation of an International Reference Preparation of Nisin by WHO in 1970. Nisin is currently approved as a food preservative in over 50 countries including the EEC (nisin's designated food additive number is E234) and the USA (Delves-Broughton 1990).

The commercial preparation, Nisaplin, contains $2^1/_2$% nisin A, the balance of material consisting of salt and milk solids derived from the fermentation of a modified milk medium by nisin producing strains of *L. lactis*. The product is standardised to a concentration of 1 million international units per gram. Recently Aplin

& Barrett Ltd, Applied Microbiology Inc have prepared high potency nisin preparations having activity of approximately 40 million international units/gram. Nisin Z, a natural nisin variant, has been described that has a substitution of His^{27} for Asn^{27} (Mulder et al. 1991). In this paper all references to nisin concentrations will be as weight of pure nisin. To convert levels of iu ml^{-1} or iu g^{-1} or to Nisaplin mg l^{-1} or mg kg^{-1} one multiplies the weight of nisin (μg ml^{-1} or g^{-1}) by a factor of 40.

Antimicrobial spectrum

Nisin shows antimicrobial activity against a wide range of Gram positive bacteria but shows little or no activity against Gram negative bacteria, yeasts or moulds (Hurst 1981). Gram positive spore formers i.e. *Bacillus, Clostridium* spp are particularly sensitive to nisin, with spores being more sensitive to nisin than vegetative cells. Such an antimicrobial spectrum has resulted in nisin being used as a commercial preservative in products which by their nature cannot be fully sterilised but only pasteurised during their production. Nisin also shows activity against lactic acid bacteria. As such bacteria are often capable of growing at low pH nisin can be used as a preservative in low pH foods that are not heat processed, such as salad dressings and alcoholic beverages. The fact that yeasts are insensitive to nisin means that nisin can be used alongside yeasts in fermentations to control the growth of lactic acid bacteria. Nisin also shows antimicrobial effect against the pathogen *Listeria monocytogenes. L. monocytogenes* is capable of growth at low temperatures and can cause severe illness and mortality among the very young, old, pregnant, ill and immunocompromised. Research has been carried out on the use of nisin as a means of elimination of *L. monocytogenes* from various foods.

Sensitivity of nisin to both vegetative cells and spores can vary between genera and even between strains of the same species.

Action of nisin against vegetative cells

Nisin works in a concentration-dependent fashion both in terms of amount of nisin applied and the number of vegetative cells or spores that need to be inhibited or killed. The primary site of action against vegetative cells is the cytoplasmic membrane, with nisin acting as a membrane depolarising agent in a voltage-dependent fashion (Henning et al. 1986a; Bruno et al. 1992; Okereke & Montville 1992). Henning and coworkers (1986a) presented evidence showing that the antimicrobial effect of nisin is caused by interaction of nisin with the phospholipid component of the cytoplasmic membrane. They demonstrated that isolated cytoplasmic membrane fragments could antagonise the inhibitory effect of nisin, and that nisin will combine with phospholipids to form nisin-phospholipid complexes. Further understanding on the mode of action of nisin, indicating that it acts on the cytoplasmic membrane forming transient pores which are dependent upon protonmotive force and membrane lipid component, has been decmonstrated by *in vitro* studies in lipsosomes (Garcerá et al. 1993; Driessen et al. 1995).

Nisin action against vegetative cells can either be bactericidal or bacteriostatic depending on the nisin concentration, bacteria concentration, physiological state of the bacteria and the prevailing conditions. Nisin will show a more pronounced bactericidal effect when conditions of test are optimum for the growth of the bacteria, e.g. optimum temperature, pH, water activity, redox potential and nutrient availability, and the bacteria are in an energised state (Sahl 1991; Maisnier-Patin et al. 1992). Conversely, bacteriostasis or inhibition of target bacteria is best achieved when nisin forms part of a multi-preservation system in which the factors above are non-optimal, or the presence of other preservatives or heat damage are also exerting inhibitory effect. Such multi-factor preservation of foods is known to food microbiologists as hurdle technology (Leistner 1994). The fact that different conditions need to be applied to ensure either destruction or inhibition of bacteria in foods needs to be taken into consideration by food microbiologists.

Abee et al. (1994) showed that with *L. monocytogenes* cells grown at 30° C, the action of nisin Z was prevented below 4° C, whereas with cells grown at 4° C, nisin Z was able to induce cell leakage at 4° C. The authors explain this phenomenon as being due to changes in the lipid hydrocarbon chains, which take place when the temperature is decreased, which results in a decrease of membrane fluidity, whereas cells grown at 4° C adapt by increasing the proportion of inactivated fatty acyl chains of the lipids which maintain membrane fluidity.

An interesting discovery is that Gram negative bacteria can be sensitised to nisin by exposure to chelating agents, sublethal heat, and to freezing (Blackburn et al. 1989; Stevens et al. 1992a; Stevens et al. 1992b; Delves-Broughton 1993). It is recognised

that Gram negative cell walls are far less permeable than Gram positive cells to various antimicrobial agents such as anionic detergents, antibiotics, lysozyme, and to various bacteriocins including nisin. Gram negative bacteria can be rendered sensitive by conversion to sphaeroplasts. Treatment of Gram negative bacteria with EDTA and other chelating agents makes them sensitive to agents they normally resist. The exact mode of EDTA and other chelating agents is to remove Mg^{2+} and Ca^{2+} ions from the Gram negative cell wall, which in turn allows the release of phospholipid and lipoprotein. The loss of these two substances increases the permeability of the cell wall and allows antimicrobial agents to act on the cytoplasmic membrane.

In a series of experiments carried out in our laboratories using mixtures of nisin and EDTA in buffer to kill the Gram negative bacterium *Pseudomonas fluorescens*, significant bactericidal effects were demonstrated (Delves-Broughton 1993). The bactericidal effect against *Ps. fluorescens* ($\log_{10}$ 6.5 cells ml^{-1}) of 0.5 mM EDTA alone and in Tris buffer (pH 7) at 30° C with an exposure time of 3 min was less than 1 log reduction of viable cells, and with 75 μg ml^{-1} nisin alone was approx. 1.5 log ml^{-1} reduction, but in combination was greater than 6.5 log ml^{-1} reduction. Interestingly, it was observed that a level of EDTA of 1.0 mM was optimum when killing bacteria on 3 minutes' exposure but that reduced kill occurred when EDTA levels were increased. Kill was significantly greater at temperatures of 20° C and above compared to temperatures below 20° C. However, when various foods were added to the test solutions bactericidal effects were greatly diminished due to preferential binding of the EDTA onto free divalent ions present in the food. The use of nisin in foods against Gram negative bacteria in combination with other agents or processes that increase cell wall permeability offers potential in the areas of food microbiology, cosmetic preservation, and veterinary and medicinal applications.

Action against spores

Mode of action against bacterial spores in the majority of cases is sporostatic rather than sporicidal. This has important implications on its use as a food preservative in heat processed foods as it means that sufficient residual nisin must be maintained throughout shelf life to provide a continued effect on any spores present. Another important observation is that spores become more sensitive to nisin the more heat damaged they are. For example, spores of the *Clostridium* anaerobe PA3679 which have survived heat treatment of 3 minutes at 121.1° C are ten times more sensitive to nisin than those which have not been heat damaged (Fowler & Gasson 1991). This effect of heat damage of spores to sensitivity to nisin has been observed with a variety of species and is an important factor in the use of nisin as a food preservative in heat processed foods.

Gould & Hurst (1962) found that spores which open their coats by mechanical rupture are more sensitive to nisin than those which do so by lysis. Spores of thermophilic bacteria such as *Bacillus stearothermophilus* and *Clostridium thermosaccharolyticum* are particularly sensitive to nisin. This has led to considerable use of nisin in canned vegetables that have a requirement for long term storage at unusually high temperatures e.g. military rations.

Nisin action against spores at the molecular level has been studied by Morris et al. (1984). They showed that nisin action against spores was caused by binding of the nisin with sulfhydryl groups on protein residues. Phospholipids were not implicated. Further work at the University of Maryland (Hansen 1994) using genetic methods has shown that nisin variants can be produced that are active against spores but not vegetative cells, and vice-versa. This clearly indicates that nisin mode of action against spores differs considerably to that against vegetative cells.

Stability and solubility

Among considerations for use of nisin for food preservation are those of stability and solubility. In a dry state, nisin concentrates are remarkably stable when protected from direct sunlight, moisture uptake, and stored at temperatures not exceeding 25° C. In solution or aqueous suspension, nisin retains its activity when heated at acid pH values (Tramer 1964). It will withstand prolonged heating at 115° C to 121° C at pH 2. In the pH range 5 to 7, nisin becomes progressively less stable to heating and significant losses in activity are to be expected when heated at elevated temperatures. Pasteurisation temperatures are less damaging to nisin and at least 80% activity will be retained, for example, during standard processed cheese manufacture. Various components in food can protect the nisin molecule to various extents during heat processing compared to the situation in buffer solutions.

Nisin is most soluble in acid substrates and becomes progressively less soluble as the pH approaches neutrality. At pH 2.2 the solubility is around 56 mg ml^{-1}, at pH 5 the value is 3 mg ml^{-1}, and at pH 11 it is 1 mg ml (Liu & Hansen 1990). In practical food preservation the levels of nisin treatment are unlikely to exceed 0.25 mg ml^{-1} and the subject of solubility is never a problem.

Antagonistic factors

Various factors in food can negate or partially negate the action of nisin. In non-heat or minimally heat processed foods proteolytic enzymes originating from microbial, plant or animal origins can degrade nisin during shelf life of the foods. As mentioned previously, nisin retention during shelf life of the food needs to be taken into consideration as action against spores is mainly sporostatic. Studies have shown that nisin retention is dependent upon the temperature of storage, the length of storage, and pH. Likely retention of nisin during shelf life is a factor dictating nisin addition levels.

As nisin is hydrophobic, fatty materials in foods can interfere with uniform distribution within the food, and make nisin unavailable for bacterial action (Jung et al. 1992). Phospholipids present in meat are suspected as causing binding of nisin, making it unavailable for bacterial action. Certain food additives have been shown to be antagonistic to nisin. For example, nisin is degraded in the presence of sodium metabisulphite (an antioxidant, bleaching and broad spectrum antimicrobial agent) and titanium dioxide (whitener) which are often used in foods. Nisin works best in liquid and homogenous foods rather than solid and heterogenous food products.

Recently, Richard (1993) found that nisin added to milk following a HTST treatment (few seconds at 72° C) had less listericidal activity compared to when milk was heated for 10 minutes at 100° C. He attributes this interesting phenomenon to possible protein changes that occur during heating: the less drastic the heat treatment, the more nisin is bound to protein. This property needs to be further investigated.

Processed cheese products

The earliest use of nisin in food was as a preservative in processed cheese products and this continues to be one of the major applications of nisin to this day (McClintock et al. 1952; Delves-Broughton 1990). The ingredients used in the manufacture of these products are raw cheese, butter, skim milk powder, often various added flavours, phosphate or citrate emulsifying salts, and added water. Spores of anaerobic clostridial species are often present in some of these ingredients, particularly the cheese, and they are usually able to survive the heat process of 85–105° C for 6–10 min which is achieved during the melt process. The composition of processed cheese in terms of the relatively high pH and moisture content combined with low redox potential (anaerobic conditions) can favour the outgrowth of these spores, which may then result in subsequent spoilage due to the production of gas, off-odours and liquefaction of the cheese. *Clostridium* species particularly associated with the spoilage of processed cheese are *C. butyricum, C. tyrobutyricum* and *C. sporogenes*. Trials with processed cheese products have been carried out in the UK using a cocktail of spores of the forementioned *Clostridium* spp at levels of approximately 200 spores per gram. In this work spoilage was prevented during storage at 37° C by 6.25 $\mu g\ g^{-1}$ nisin, partial control was achieved with 2.5 $\mu g\ g^{-1}$ nisin whilst the control samples readily spoilt.

The potential for growth and toxin production by *C. botulinum* in processed cheese products, particularly spreads, is of considerable significance. Trials have indicated that nisin is effective in these spreads in delaying or preventing the growth and subsequent formation of toxin by inoculated spores of *C. botulinum* types A and B (Somers & Taylor 1987).

Addition levels of nisin to achieve effective preservation depend on the following factors: the spore load present in the formulation, moisture content, pH, salt content, use of flavour additives, cooking process employed and the length and likely temperature of the shelf life required. Levels used to prevent spoilage vary from 5 to 20 $\mu g\ g^{-1}$, whereas levels used to provide protection against *C. botulinum* are 12.5 $\mu g\ g^{-1}$ and higher.

Other pasteurised dairy products (Delves-Broughton 1990)

In countries with high ambient temperatures and inadequate refrigeration or transport facilities, nisin can be usefully employed in pasteurised milk at levels of 0.625–1.25 $\mu g\ ml^{-1}$ to provide significant increases in shelf life. Effective preservation of pasteurised

flavoured milks and dairy desserts has also been reported using levels of 2.5–3.75 $\mu g\ ml^{-1}$.

Canned vegetables (Delves-Broughton 1990)

Low-acid canned foods (pH above 4.5) should receive a minimum heat process of Fo = 3 to ensure the destruction of *C. botulinum* spores, i.e. the minimum botulinum cook. Low-acid foods processed at Fo of 3 and above are, however, still susceptible to spoilage from surviving heat-resistant spores of the thermophilic bacterial species of *B. stearothermophilus* and *C. thermosaccharolyticum*. Thus, nisin addition to low-acid canned vegetables can facilitate prolonged storage of these products at warm ambient temperatures by controlling the growth of these thermophilic spoilage organisms. The use of nisin can also permit a reduction of the Fo process down to the minimum of 3 without increasing the potential risk of thermophilic spoilage.

Bacterial spoilage of high-acid foods (pH below 4.5) is restricted to non-pathogenic, heat-resistant, aciduric, spore-forming bacterial species such as *Clostridium pasteurianum, Bacillus macerans*, and *Bacillus coagulans*. Spoilage resulting from the growth of these bacteria can be effectively controlled by nisin.

High moisture hot plate bakery products (crumpets) (Jenson et al. 1994)

Crumpets are high moisture, flour based products that are particularly popular in the United Kingdom and Australia. They are produced on a hot plate from a flour batter and contain yeast, an aerating agent or both to give them a raised profile and open texture. They are traditionally toasted before consumption. Crumpets have a non-acid pH (6–8), high moisture (48–54%) and high water activity (0.95–0.97). The product is sold at ambient temperature.

There have been a number of outbreaks of food poisoning due to growth of *Bacillus cereus* in crumpets. Flour used in the manufacture of crumpets will invariably contain a low number of *B. cereus* spores. In the cooking process the bottom of the crumpet receives a high heat treatment but the rest of the crumpet receives a lower heat treatment which *Bacillus* spores easily survive. During the 3–5 day shelf life of the product the levels of *B. cereus* may increase from undetectable levels to greater than $10^5\ g^{-1}$ which can be sufficient to cause food poisoning.

Research carried out in Australia and the U.K. has shown that *B. cereus* isolated from untreated crumpets at the end of shelf life were shown to be sensitive to nisin. Addition of nisin to batter at levels of 3.75 $\mu g\ g^{-1}$ and above effectively prevented the growth to levels capable of causing food poisoning. This research and subsequent factory trials resulted in regulations in Australia allowing the use of nisin in crumpets and similar high moisture flour based products.

Pasteurised liquid egg products (Delves-Broughton et al. 1992)

Pasteurised liquid egg products (whole, yellow and white) receive heat treatments designed to ensure the destruction of Salmonella. These are typically 62–65° C for 2 to 3 minutes. However, such heat treatment is insufficient to kill off bacterial spores and some species of both Gram positive and Gram negative bacteria. Many of these surviving bacteria are capable of growth at refrigerated temperatures and pasteurised liquid egg products usually have a limited shelf life. Application of nisin at levels of 2.5 and 5 mg l^{-1} has shown to act as an effective preservative giving significant increase in shelf life and providing protection against the growth of psychrotrophic *B. cereus*. Such use of nisin is of particular interest in the U.S.A. in modified egg products that have greatly reduced cholesterol levels. Further unpublished trials indicate that nisin is more effective in liquid white compared to liquid yellow. This could be due to the high level of phospholipid present in the yellow interfering with the mode of action of nisin, and to the presence of other antimicrobial factors in liquid white such as ovotransferrin and lysozyme. Extracted lysozyme from egg white has been shown to be synergistic with nisin in terms of bacteriostatic activity, and the combination possesses wider antimicrobial spectrum than either agent used alone (Monticello 1989).

Meat

Concern on high levels of nitrite in cured meat has led various workers to consider alternative preservation systems, which include a reduction in nitrite levels, and these have included nisin (Caserio et al. 1979a; Caserio et al. 1979b; Rayman et al. 1981; Rayman et al. 1983;

Calderon et al. 1985; Houben & Krol 1985; Taylor & Somers 1985; Taylor et al. 1985). Results indicated that only high levels of nisin, which would be uneconomic, achieved good control of *Clostridium botulinum* and that further work is required before a case can be proposed for the use of nisin as a partial replacement for nitrite. More encouraging results have been obtained in vacuum packed cured meats and sausages where the limiting factor to shelf life can be the growth of lactic acid spoilage bacteria. Consideration needs to be taken in some meat systems that the effect of nisin does not produce an 'ecological vacuum' which may be exploited by other competing micro-organisms that are normally suppressed.

Alcoholic beverages

Research in Europe has demonstrated that nisin has potential in controlling spoilage lactic acid bacteria in beer (Ogden & Tubb 1985; Ogden 1986; Ogden & Waites 1986; Ogden 1987; Ogden et al. 1988) and wine (Radler 1990a, 1990b). The studies indicate that although the spoilage lactic acid bacteria are sensitive to nisin, the yeasts are completely unaffected. This important factor allows the nisin to be introduced during the fermentation. Applications identified in the brewing industry are: adding to fermenters to prevent or control contamination; washing pitching yeasts to eliminate contaminating bacteria as an alternative method to acid washing which is known to affect yeast viability; reducing pasteurisation processes; and increasing the shelf life of unpasteurised or bottle conditioned beers. Similar applications occur in the wine industry. Nisin cannot be used during the fermentation in wines that depend on a desirable malolactic acid fermentation.

Nisin can also be used in distilled alcohol production, both for beverages and industrial products. Added to fermentation mashes naturally contaminated with lactic acid bacteria, the latter's activity can be controlled, allowing the yeast less competition for substrate, thus resulting in increased alcohol yield (Henning et al. 1986b).

Salad dressings

Investigations are underway at Indiana State University on the use of nisin to control lactic acid bacteria spoilage of salad dressings. There is a requirement in the USA for the development of salad dressings with less acidity and this gives improved flavour and appreciation of added flavour ingredients. Raising the pH from 3.8 to 4.2 can make salad dressings prone to spoilage by lactic acid bacteria during ambient storage. Such growth has been successfully controlled by the addition of nisin at levels of 2.5 to 5 mg l^{-1}.

Natural cheese

Use of nisin producing starter cultures to manufacture cheese with significant levels of nisin are being investigated by a number of research groups. Natural cheese products are sensitive to infection by several different Gram-positive micro-organisms. Many soft, surface-ripened, cheese types are very sensitive to infection with *Listeria monocytogenes* and unacceptably high numbers of this micro-organism have been found in these cheeses. In cheeses made from fresh, unheated milk, different endogenous *Lactobacillus* species can cause off-flavours and gas production. Cheese made under more artisanal conditions, in dairy farms for instance, are usually infected with undesirable amounts of *Staphylococcus aureus*. In large scale production of Gouda cheese and Emmental cheese the main bacterial problem is butyric acid fermentation caused by *Clostridium tyrobutyricum*. Nisin has antibacterial activity against all these undesirable micro-organisms in cheese. Currently, especially in large industrial productions, other agents such as sodium nitrate are routinely added to the cheese milk for the necessary protection. Since this has become increasingly unpopular, and usually only works against specific micro-organisms (such as *Clostridium*) nisin is considered an attractive alternative. Since direct addition of nisin to cheese-milk is costly, inefficient and prohibited for the production of most natural cheese-types, the use of nisin-producing starters is the obvious strategy. However, no existing nisin-producing starters have the flavour-generating, eye-forming, acidifying activities and bacteriophage-resistance which are suitable for the manufacture of most cheese types. For the production of Gouda a special nisin-producing starter was developed. Since the ability to produce nisin is encoded on a transposon (Rauch & de Vos 1992), a mobile DNA element, it was decided to transfer this trait to industrial strains. These strains were carefully selected from the complex mixed starter cultures that are used in the Dutch dairy industry. Several combinations of a proteolytic *Lactococcus lactis* subsp. *cremoris* strain

and a citrate-utilising *Lactococcus lactis* subsp. *lactis* (bv. diacetylactis) performed equal or better as cheese-starter than the popular mixed starter BOS which is used most frequently for Gouda production in the Netherlands. Nisin-production was introduced in these strain combinations by conjugative transfer of nisin-immunity to the proteolytic strain and nisin-production to the citrate-utiliser. By using lactose-negative strains as donor transconjugants could be directly selected. These were carefully analysed for retention of all important faculties such as bacteriophage-resistance, proteolytic or citrate-utilising activity and acidification rate. Furthermore, the transconjugants were again tested in combination for their suitability for cheese making. Finally, concentrated starters were developed from a selection of successful transconjugants as pure cultures. By using the nisin-producing and nisin-immune strain in different ratios as starter, cheese could be produced with various levels of nisin. The nisin-containing cheese showed protection against infection of *Clostridium tyrobutyricum* and *Staphylococcus aureus* during the whole period of ripening.

Antilisterial properties of nisin

Increasing concern about food-borne listeriosis has prompted the evaluation of nisin both as a bactericidal and bacteriostatic agent against *L. monocytogenes*. As mentioned previously it is evident that conditions that result in nisin exerting a strong bactericidal effect differ from those that exert a strong bacteriostatic effect.

Various workers have shown an initial reduction of *Listeria* numbers can be achieved by application of nisin in microbiological media but that on subsequent incubation can be followed by growth to population levels that are eventually similar to those of the controls. Mohamed et al. (1984), working with two strains of *L. monocytogenes*, showed that nisin levels ranging from 0.2 to 0.4 mg l^{-1} caused an initial reduction from 10^5 cfu ml^{-1} to less than 10^1 cfu ml^{-1} in broth, followed by subsequent growth of survivors to a level similar to the control, but at levels above 8 mg l^{-1} complete inhibition was achieved. Richard (1993) found that nisin at 0.5 mg l^{-1} caused a reduction from 10^7 to 10^5 cfu ml^{-1} with subsequent growth again occurring. It was also observed that death (Dmax) was optimum at pH 6 and 7 compared to pH 4 and 8. Harris et al. (1991), using a direct plating method, showed that nisin at 10 μg ml^{-1} reduced a 10^9 cfu ml^{-1} culture of *L. monocytogenes* down to 10^2–10^3 cfu ml^{-1}. Sensitivity to nisin was enhanced by addition of 2% NaCl or by reduction of the medium pH from 6.5 to 5.5. Mutants resistant to nisin at 50 mg l^{-1} were detected at frequencies of 10^{-6} to 10^{-8}. A similar rate of resistance has been reported elsewhere. Mechanism of nisin resistance has been shown to be due to differing absorption rates of nisin onto cell surfaces with more sensitive cells able to absorb more nisin (Davies & Adams 1994). Research by Ming & Daeschel (1993) indicates that resistance is due to fundamental changes occurring in bacterial cytoplasmic membrane structure and function as opposed to a resistance response involving nisin degradation. Ferreira & Lund (1991) compared the sensitivity to nisin of 27 strains of *L. monocytogenes* of several serotypes, and strains of *L. innocua* and *L. ivanovi* by determination of the MIC values at pH 5.5 at 20° C and found MIC value of the majority of strains was 2.5 to 10 mg l^{-1}. A representative resistant strain of *L. monocytogenes* was rapidly killed in culture medium at pH 5 containing 12.5 mg nisin l^{-1} and incubated at 20° C whereas the organism multiplied in these conditions in the absence of nisin. In cottage cheese with pH 4.6 and 5.0, nisin had a lower listericidal activity than in culture medium.

Jung et al. (1992) showed that the presence of milk fat affected adversely the antilisterial property of nisin against *L. monocytogenes*. A concentration of nisin of 1.25 mg l^{-1} was very effective in achieving a four to six log reduction in non-fat milk whereas in half and half cream less than one log reduction was achieved. Contrary results to this were obtained by Richard (1993) who showed that nisin at 20 mg l^{-1} applied for 4 hours at 15° C gave comparable kills in skim to 3.2% fat milk. This discrepancy was explained by the physical condition of the fat. In fresh raw milk, fat is nearly always present in intact globules, whereas in pasteurised milk of less good bacteriological quality, more free fat and fatty acids are present.

Workers at INRA in France have devised a system controlling the growth of *Listeria* in the production of surface ripened soft cheese. The system involves adding nisin to the raw milk at levels ranging from 0.625 to 2.5 mg l^{-1}, followed by a mild heat treatment, and then the addition of nisin producing nisin resistant starter cultures. Such an approach results in reduction of 5 to 6 log cycles of inoculated *L. monocytogenes* (Richard 1993).

The use of nisin in combination with reduced heat processes has been studied by other research groups. Heat processing of canned lobster which is retailed frozen can only be achieved by heating at 60° C for 5 minutes without adverse product shrinkage occurring. Such heat processing results in a 2 log reduction of *L.*

monocytogenes whereas addition of nisin to the brine at 25 mg l^{-1} increases the log reduction from 5 to 6. Mahadeo and Tatini (1994) working with turkey poultry showed that nisin at 2.5 mg l^{-1} provided a synergistic effect to the kill of *L. monocytogenes* by heat (52° C for 3 minutes) in the scald water but the effect was less pronounced when the bacteria were associated with the turkey skin.

The control of growth of *L. monocytogenes* on cooked pork tenderloins has been studied in Taiwan (Fang & Lin 1994). Control was achieved using 250 mg l^{-1} surface treatment. The inhibitory effect of nisin in a modified atmosphere system was more pronounced at 4° C than at 20° C. Washing blue crab (*Callinectes sapidus*) with nisin at levels of 2.5 to 5 mg l^{-1} caused log reduction of inoculated *L. monocytogenes* cells of approximately 2 to 3 log cycles respectively (Degnan et al. 1994).

Further studies are underway in the antilisterial effect of nisin. It is apparent that the chemical composition of food is an important factor, as some food constituents appear to have the capacity to bind nisin.

Combination treatments

There is considerable interest in using nisin in combination with novel non-thermal preservation techniques such as ultrahigh hydrostatic pressure (UHP) and pulsed electric field (EP) techniques. Kalchayanand et al. (1994) report that injured cells of *L. monocytogenes, E. coli* and *S. typhimurium* following such treatment were sensitive to nisin, and that nisin in combination with either UHP or EP had greater antibacterial effectiveness than UHP or EP alone. Such combination treatments are also being carried out on spores.

In an extension of the studies carried out on nisin in combination with chelating agents, ter Steeg (1993) and ter Steeg et al. (1994) have investigated the combination of nisin, lysozyme and citrate. Synergistic and additive effects were apparent against *L. monocytogenes* and a combination treatment successfully prevented the growth of *L. monocytogenes* in liver paté, and *L. monocytogenes* and spoilage bacilli in processed cheese. Use of citrate alone, lysozyme and citrate, or nisin and citrate failed to prevent growth of the inoculated bacteria.

Pharmaceutical and veterinary applications

The preparation of highly purified nisin and the observation that both the level and spectrum of activity can be considerably enhanced by combination with chelating agents (Blackburn et al. 1989) have each opened up a number of veterinary and pharmaceutical applications for this bacteriocin.

One potential medical application is as a therapy for the cure of peptic ulcer disease, a chronic inflammatory condition of the stomach and duodenum affecting some 3–10% of the population. Standard therapy involves treatment with agents that reduce gastric acid secretion. However, despite high apparent cure rates, the condition frequently recurs. In February 1994 the National Institute of Health issued a consensus statement recognising a causative link between peptic ulcer disease and colonisation by the Gram negative bacterium *Helicobacter pylori*. The bacterium can be eradicated by antibiotic treatments, though these often involve complex regimens leading to poor patient compliance. There is also an increasing incidence of bacterial resistance and the problem of side effects due to the activity of the antibiotics against commensal gut microflora.

Helicobacter pylori has been shown to be sensitive to nisin *in vitro*, this sensitivity being considerably enhanced by the presence of a chelator. Nisin is well-suited as a therapeutic for use in the human stomach and duodenum due to its preference for low pH and insensitivity to the stomach protease, pepsin. Importantly, nisin is rapidly degraded by pancreatic proteases and so would not be expected to survive into the high pH environment of the small intestine. This reduces the potential for gastrointestinal side effects. Other properties of nisin that suggest it will be a safe, effective therapeutic agent for the eradication of *H. pylori* include its long history of use in food, its low toxicity and its rapid bacteriocidal activity. The last property (in which it differs from most antibiotics which are bacteriostatic), and the failure of nisin to reach the microflora of the gut, should decrease the potential for the development of acquired resistance. This is a key difference between peptide antimicrobials such as nisin and the antibiotics. Taken together, the properties of nisin suggest not only that it has potential for the treatment of peptic ulcer disease but that it may also have application as a prophylactic agent in the prevention of *H. pylori* colonisation progressing to a disease state. This therapeutic application of nisin has been patented (Blackburn & Projan 1994).

Nisin also shows good potential as a therapeutic agent in the treatment of bovine mastitis. At present,

the condition is commonly treated by the intramammary administration of antibiotics. During lactation however, this approach has a low cure rate and the presence of drug residues can lead to prolonged withholding periods. Nisin is strongly bacteriocidal towards mastitis pathogens (*Staphylococcus* and *Streptococcus* species). In field trials, intramammary infections were produced by challenge with an intramammary inoculation or by teat exposure to the pathogen. Following just three intramammary treatments at 12 hour intervals high cure rates were achieved (66% for *Staphylococcus aureus*, 95% for *Staphylococcus agalactiae* and 100% for *Streptococcus uberis*). There was no correlation between the number of days infected and the cure rate. Following treatment, a significant reduction of somatic cells was observed in glands scored as cured by microbiological criteria. The experimental trial suggests that, appropriately formulated, nisin could be very useful in the treatment of bovine intramammary infections. Because nisin is an antimicrobial peptide rather than an antibiotic, is non toxic and readily inactivated by digestive enzymes in the gut, it should be possible to demonstrate that the agent and its peptide residues pose no hazard to the milk supply.

The characteristics of the nisin molecule which make it suitable for the applications described above also apply to a number of other current and potential opportunities in the veterinary and pharmaceutical areas. Nisin is already being used as a preventative agent against bovine mastitis through its use in pre- and post-milking teat dip products. A number of oral care applications are also being actively explored. A nisin-based mouth rinse was evaluated in a beagle dog model, and was shown to prevent the build-up of plaque and to prevent gingival inflammation (Howell et al. 1993). The exquisite sensitivity of *Streptococcus* and *Staphylococcus* species to the nisin offer opportunities in areas such as topical skin infections and the treatment of multiple drug resistant (MRSA) systemic infections.

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