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Interactions of Nisin with Meat Components and their Effect on Activity

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A thesis submitted to the University of Surrey in part-fulfillment of the requirements for the degree of Doctor of Philosophy
To my parents
Thanasis and Elli Stergiou

Στον/σα γονείς μου
Θανάση και Ελλή Στεργιού
SUMMARY

Nisin is a bacteriocin produced by Lactococcus lactis subsp. lactis, with antimicrobial activity against many Gram-positive bacteria, including bacterial spores. Nisin has been successfully used in numerous products, such as processed cheeses, dairy products and canned foods. However, its effectiveness in meat products is variable due to interactions with certain meat components. The aim of this project therefore was to determine a number of factors reported to reduce nisin activity in meat and meat products, evaluate the extent of interference with its efficacy and introduce multicomponent preserving systems that improve its antimicrobial action.

Loss of nisin activity in meat has been partly ascribed before to the formation of a nisin-glutathione adduct. Activity is lost more quickly in raw meat than in cooked meat and this has been taken as evidence that the reaction is enzyme-mediated. Horizontal agar diffusion method and MALDI-TOF MS confirmed the formation of the nisin-glutathione adduct which was shown not to be enzyme mediated. Retention of activity in cooked meat was shown to be due to loss of free sulfhydryl groups during heat processing as a result of the reaction of glutathione with proteins and not a result of the inactivation of endogenous enzymes, as indicated by measuring the free thiol content. Microbial enzymes did not appear to play a role as similar losses were seen in raw and cooked meat extracts containing undetectable levels of microorganisms.

Enumeration of viable counts indicated that increased nisin concentrations were required for the total inhibition of L. monocytogenes following incubation of nisin with glutathione. The inhibitory effect of glutathione on nisin activity was removed in the presence of ascorbic and dehydroascorbic acid, sometimes with enhancement of nisin’s bactericidal effect. However, there was no evidence for a direct reaction between glutathione and dehydroascorbic acid. The addition of ascorbic acid and dehydroascorbic acid in raw meat extract as well as in raw and thermally processed meat slurry was shown to exert the same protective effect seen with laboratory media. The synergy between nisin, ascorbic and dehydroascorbic acid was more pronounced at chill temperatures.

Simultaneous application of antioxidants and nisin delayed the recovery of nisin-injured L. monocytogenes cells especially at low temperatures. Antioxidants, at the levels used,
had no bactericidal effect on their own, but they did retard growth by decreasing growth rate and increasing lag phase. This was probably due to antioxidants diverting energy from injury repair-related functions. The effectiveness of the combination of nisin with propyl gallate was completely negated in whole fat UHT milk due to interactions of nisin with fat.

Overall, the findings of this study clearly show that nisin performs better when its use is supplemented with other preservation agents or methods, since its amphiphilic properties make it vulnerable to interactions with other food macromolecules resulting in reduced efficacy.
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ABBREVIATIONS

AA: Ascorbic Acid
ADSM: Alkalase Digested Skimmed Milk
ANOVA: Analysis of Variance
ATCC: American Type Culture Collection
ATP: Adenotriphosphoric acid
AU: Arbitrary Units
BHA: Butylated hydroxyanisole
BHI: Brain Heart Infusion
BHT: Butylated hydroxytoluene
BSA: Bovine Serum Albumin
CFU: Colony Forming Units
Dha: Dehydroalanine
DHAA: DeHydroAscorbic Acid
Dhb: Dehydrobutyrate
DPPH: 2, 2-diphenyl-1-picrylhydrazyl
DTNB: 5,5'-dithiobis’2-nitrobenzoic acid
DTT: Dithiolthreitol
EDTA: Disodium EthyleneDiamineTetra-Acetic acid
GSH: Glutathione
GSSG: Oxidised glutathione
GST: Glutathione S-Transferase
HPLC: High-Performance-Liquid Chromatography
IU: International Units
LSA: Listeria Selective Agar
MALDI-TOF MS: Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry
MIC: Minimum Inhibitory Concentration
MRD: Maximum Recovery Diluent
NB: Nutrient Broth
NCIB: National Collection of Industrial Bacterial
NCTC: National Culture Type Collection
PG: Propyl Gallate
RE: Rosemary Extract
SN: Sodium Nitrite
UHT: Ultra High Temperature
VTEC: Verocytotoxin-producing *E. coli*
WHO: World Health Organisation
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CHAPTER 1
GENERAL INTRODUCTION
CHAPTER 1

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1.1 NATURAL PRESERVATION OF FOOD SYSTEMS

Changes in people's lifestyles in recent years have had considerable impact on their food purchasing behaviour as well as on their eating habits and preferences. On the one hand, consumers demand convenience and on the other, products that are less heavily preserved, less processed, free from "artificial" additives, fresh and more natural but with assured safety and keepability. Public concerns over possible health hazards posed by food additives, and suspicion that poor hygiene and sloppy manufacturing practices are masked by the addition of preservatives have forced most regulatory authorities to oppose extension of the use of food preservatives (Bell and De Lacy, 1987).

Although minimally processed foods of extended durability, refrigerated or not, satisfy consumer demand for foods that are "fresh", "natural", and "preservative free", the potential for the growth of pathogenic microorganisms remains a concern.

Microbiological stability and safety of food products are essential for the development of national and international food trade. To sustain market developments and to certify quality, the selection of suitable preservatives must be based on sound assessments of the risks and benefits associated with their use in specific products (Bell and De Lacy, 1987).

As natural preservation agents are felt to be compatible with both commercial requirements and public and regulatory concerns, there has been a considerable interest in what are described as natural food preservatives (natural antimicrobial substances produced by plants, animals and microorganisms).
1.2 BACTERIOCINS: A GENERAL APPROACH

Antimicrobial polypeptides produced by food-grade starter culture bacteria, known as bacteriocins, have been the centre of attention in recent years because of their possible use as natural food preservatives in place of some of the currently used preservatives. Bacteriocins are ribosomally synthesised and their most pronounced difference from traditional antibiotics is the fact that they usually only kill bacteria which are closely related to the producing strain. The bacteriocins were first characterised in Gram-negative bacteria, especially the colicins produced by *Escherichia coli* (*E. coli*) which have been extensively studied (Cleveland *et al.*, 2001). Among the Gram-positive bacteria, the lactic acid bacteria have been systematically investigated as a source of bacteriocins.

Bacteriocins are commonly classified into three major categories:

a. Class I or lantibiotics, including nisin and subtilin. This group is further divided into two subgroups Ia and Ib. The former consists of cationic and hydrophobic peptides that form pores in target membranes and have a flexible structure as compared with Class Ib bacteriocins, which include globular polypeptides that have no net charge.

b. Class II contains small heat-stable, non modified peptides. This category is again divided into two subgroups. Class IIa includes Pediocin-like *Listeria* active peptides with a conserved N-terminal sequence Tyr–Gly–Asn–Gly–Val and two cysteines forming an S–S bridge in the N-terminal half of the peptide. Bacteriocins belonging to Class IIb group consist of two different peptides and both are required to form an active poration complex.

c. Class III bacteriocins are large molecules demonstrating heat sensitivity (Cleveland *et al.*, 2001).

The centre of interest of the present study is nisin which is a member of the Class Ia bacteriocins.
1.3 NISIN

Nisin is a polypeptide produced by certain strains of the food grade lactic acid bacterium *Lactococcus lactis* subsp. *lactis*. Unlike most bacteriocins, nisin exhibits a relatively broad spectrum of activity. It is active against a wide range of Gram-positive vegetative bacteria, and is particularly effective against bacterial spores (Davies and Delves-Broughton, 1999). Gram-negative bacteria, moulds and yeasts are generally resistant.

1.3.1 History of nisin

Nisin was first discovered in the 1920s, when problems arose during cheese production. Batches of milk starter cultures used in the process were contaminated with a nisin producing strain of *Lactococcus lactis* and the development of cheese was seriously affected (Delves- Broughton, 1990). Nisin was named from group N (Streptococci) Inhibitory Substance (Mattick and Hirsch, 1947). Initially, nisin was investigated for medical and veterinary purposes, but characteristics such as low solubility at neutral pH and its immediate digestion made it unsuitable for use.

However, its digestibility and heat stability at low pH did make it an ideal candidate for a food preservative. Hirsch (1951) showed that clostridial gas formation in cheese was prevented by nisin-producing starter cultures, and that was the first evidence for the use of nisin as food preservative. Later, numerous applications were identified (see later, section 1.3.7), and in 1969, nisin was approved for use as an antimicrobial agent by the Joint Food and Agriculture Organisation/World Health Organisation Committee on Food Additives (Delves-Broughton, 1990).

1.3.2 Structure and biosynthesis

Nisin belongs to the Ia subgroup of bacteriocins known as lantibiotics, which are produced by various Gram-positive bacteria, such as *Staphylococcus*, *Lactococcus*, *Streptococcus*, *Bacillus* and *Streptomyces* (Jung, 1991). Lantibiotics are relatively small polycyclic polypeptides, which contain the unusual amino acids lanthionine and/or β-methylanthionine, both of which form intrachain thioether bridges (de Vos et al., 1991).
Nisin's structure was first clarified by Gross and Morell (1971). It consists of 34 amino acids with a molecular weight of 3354 Da and, in addition to lanthionine and β-methyllanthionine it also contains another two unusual amino acids, dehydroalanine and dehydrobutyrine (Fig. 1.1). These arise from the dehydration of serine and threonine respectively, and the condensation of Dha and Dhb with cysteine generates thioether bonds and the amino acids lanthionine and β-methyllanthionine respectively (Davies and Delves-Broughton, 1999). The thioether bonds give nisin two rigid ring systems, one located at the N-terminus and the other at the C-terminus, which maintain the molecule in a screw-like conformation (Breukink and de Kruijff, 1999).

Figure 1.1 The primary structure of the lantibiotic nisin Z. The unusual residues are dark gray for lanthionine residues and light gray for dehydrated residues. Dha, dehydroalanine; Dhb, dehydrobutyrine; Ala-S-Ala, lanthionine; Abu-S-Abu, β-methyllanthionine

From Breukink and de Kruijff, 1999.

Nisin is synthesized ribosomally as a precursor peptide, which is then enzymatically cleaved (to give pronisin) and post translationally modified to generate the mature lantibiotic (Davies and Delves-Broughton, 1999) (Fig. 1.2). There are two naturally occurring nisin variants that have similar activities, nisin A and nisin Z. They differ only
in the amino acid residue in position 27, which is asparagine in Nisin Z instead of histidine (Nisin A) (Breukink and de Kruijff, 1999).

1.3.3 Nisin properties

Lanthionine is known to introduce a high level of hydrophobicity, and the high proportion of basic amino acids also gives nisin a positive charge (+4). In aqueous solution, nisin is most soluble in pH 2, whereas at higher pH values, water solubility decreases. The presence of nucleophiles makes Dha and Dhb susceptible to modification, which may explain the decreased solubility and instability of nisin under basic conditions (Davies and Delves-Broughton, 1999). Limited solubility does not, however, appear to be a practical problem, as even at neutral pH its solubility exceeds the levels usually necessary for activity in foods.

Of great significance is the fact that nisin is heat stable, particularly at acidic pH values. It has been shown to lose less than 5% of its activity during autoclaving (115°C for 20 min) at pH 3 and pH values below or above this range can cause a more marked decrease (Davies et al., 1998). However the impact of neutral or higher pH on nisin stability is less apparent at pasteurization temperatures (Davies and Delves-Broughton, 1999), and thus, nisin can be used successfully in such foods, taking into consideration that the food substrate itself will also protect the nisin molecule (Heinemann et al., 1965).

High salt concentrations (appr 10%) and their effect on the stability of nisin solutions have also been assessed. It has been reported that after 24 h incubation at ambient temperature, nisin loss due to precipitation increased with an increase in the level of NaCl added. The effect was more significant in high concentration nisin solutions (>2000 IU/ml) (Thomas et al., unpublished data). Nevertheless, this does not comprise a major practical problem for the food manufacturers since nisin is added in lower concentrations and directly as a powder.
Figure 1.2 Nisin biosynthesis. NisA: pre nisin structural protein; NisB: maturation enzyme; NisT: transport protein; NisC: maturation enzyme; NisI: nisin immunity protein; nisP: proteinase cleaves leader; NisR: regulatory protein; NisK: regulatory protein; nisFEG: nisin immunity proteins

1.3.4 Mode of action and antimicrobial properties of nisin

Nisin’s antibiotic activity is based on a multiplicity of activities which may combine differently for individual target bacteria, and that could explain the fact that, in contrast with other bacteriocins, its spectrum of activity is not confined to closely related species.

Much focus has been given to the way nisin acts on vegetative cells. The first report on the mode of action of nisin appeared in 1960, when Ramseier described a detergent-like membrane disruption based on the observation that nisin-treated bacteria leaked UV-absorbing cellular material (Ramseier, 1960). Gross and Morell (1971) attributed the antimicrobial activity of nisin to the reaction between the molecule’s dehydro-residues and intracellular enzyme sulfydryl groups. Other studies revealed that the antimicrobial effect of nisin was caused by inhibition of cell wall synthesis either by the interaction of the lantibiotic with the membrane-bound cell wall precursors, lipid I and lipid II (Reisinger et al., 1980) or, by direct interaction with phospholipid components of the cytoplasmic membrane, followed by destruction of the membrane function (Henning et al., 1986). The latter worker also demonstrated that nisin inhibits the synthesis of murein, a cell wall component, but the sensitivity to nisin of microorganisms lacking that component, showed that the inhibition of murein synthesis is not a primary mode of action.

It is generally accepted that nisin’s effect is through pore formation in the bacterial plasma membrane. Pore formation causes rapid efflux of small cytoplasmic compounds, collapse of vital ion gradients and dissipation of the membrane proton motive force, resulting in cell death (Breukink and de Kruijff, 1999).

Two main theories have been formulated concerning pore formation. It has been suggested that nisin follows the barrel-stave model of pore formation (Hechard and Sahl, 2002), where peptides first adopt a transmembrane orientation before they aggregate to form water filled pores, but this model had to be abandoned as it would imply exposure of charged groups in the lipid phase of the bilayer, which is energetically highly unfavourable.
The basic character of nisin suggests that it preferentially interacts electrostatically with negatively charged membranes, thus with membranes that have relatively high concentrations of anionic lipids (Breukink and de Kruijff, 1999). The plasma membrane of Gram-positive bacteria contains a higher proportion of anionic lipids than that of the Gram-negative bacteria (O’Leary et al., 1988). This could partially explain the greater sensitivity of Gram-positive bacteria to nisin.

The C-terminus seems to play an important role in the binding of nisin to the target membrane due to its positive charge, whereas the amphiphilic properties of the peptide allow it to insert into the lipid phase of the membrane. The N-terminus of the molecule primarily inserts into the lipid phase of the membrane due its high hydrophobicity. The nisin molecule is then stably orientated parallel to the membrane surface (Breukink and de Kruijff, 1999). Upon insertion into the membrane, interaction with phospholipid head groups causes a locally disturbed bilayer structure, while the hydrophobic residue inserts into the membrane (Fig. 1.3).

This model, known as the “wedge model”, could explain the killing activity of microbes that have micromolar minimum inhibitory concentrations (MICs). On the other hand nisin antibacterial activity in nanomolar concentrations may indicate that additional activities or specific targets may be involved (Fig 1.4). In vitro studies have shown that lipid II serves as a docking molecule for specific binding to bacterial membrane (Hechard and Sahl, 2002).

In addition to its activity against Gram-positive vegetative cells, its ability to prevent the outgrowth of germinating bacterial spores by inhibiting pre-emergent swelling is of vital importance (Davies and Delves-Broughton, 1999). Its activity against spores is enhanced under acidic condition or if the spores have been injured (Adams and Smid, 2003). Gould and Hurst (1962) first observed that spores that rupture their coats mechanically are more sensitive than with those spores which do so by lysis. Studies on the action of nisin against spores at the molecular level revealed that inhibition of outgrowth was due to reaction of nisin’s dehydro-groups with membrane-bound sulfhydryl groups present in newly germinated spores (Morris et al., 1984). Substitution of the dehydroalanine residue at position 5 by an alanine residue caused reduced inhibition of spore outgrowth but did
Figure 1.3 Model of the mode of action of nisin. Binding of nisin via its C-terminus (step I), insertion of nisin into the membrane (step II), pore formation by nisin (step III) in which the right part represents the situation when phospholipids are active members of the pore, and translocation of the whole peptide (step IV).

From Breukink and de Kruijff, 1999

not reduce the activity against vegetative cell (Chan et al., 1996). Sensitivity to nisin may vary considerably between mesophilic and thermophilic spores, the former being more resistant, or between spores produced by different strains of the same genus (e.g. Clostridium).

In general, nisin action against spores is mainly bacteriostatic, rather than bactericidal, and this should be considered as a key issue when it comes to the preservation of heat-treated food products. Sufficient amounts of nisin have to be retained throughout the shelf-life of the product to provide a continuous effect on any spores present, though it has been shown that heating of spores results in increased sensitivity to nisin (Hall, 1966; Heinemann, 1965).
Figure 1.4 Molecular activities of type-A lantibiotics (nisin). (a) at micromolar concentrations they form wedge-like, target independent pores, (b) at nanomolar concentrations, they form target-mediated pores using lipid II as a docking molecule. From Hechard and Sahl, 2002.

Gram-negative bacteria are nisin resistant, as their protective outer membrane excludes the free passage of molecules exceeding 700 Da (Adams and Smid, 2003). However, increased permeability of their outer membrane can be achieved using chelating agents which remove magnesium ions that stabilize the lipopolysaccharide layer of the outer membrane. Stevens et al., (1991) achieved significant kill of Salmonella and other Gram-negative species of bacteria, by combining nisin with disodium ethylenediaminetetra-acetic acid (EDTA). Furthermore, it has been reported that Gram-negative bacteria sensitivity to nisin can also be increased by combining the polypeptide with heating, freezing or exposure to organic acids (Delves-Broughton and Gasson, 1994; Boziaris and Adams, 2001).
1.3.5 Nisin resistance

A number of studies have particularly focused on the antilisterial effect of nisin. *Listeria monocytogenes* is difficult to control in foods because of its ability to grow at refrigeration temperatures, relatively low pH values and high NaCl concentration. *Listeria monocytogenes* strains show considerable variation in their nisin sensitivity. Mohamed *et al.*, (1984) observed that the Minimum Inhibition Concentrations (MICs) varied depending upon the strain evaluated, and that at lower nisin concentrations further incubation resulted in maximum bacterial numbers. Harris *et al.*, (1991) found that nisin had bactericidal effect on *L. monocytogenes* ATCC 19115, Scott A, and UAL500 when it was added at a level of 400 IU/ml. Susceptibility of the microorganism was enhanced by combining nisin with other growth barriers such as NaCl (2.5%), and low pH. Resistance to nisin by mutant *Listeria* strains has been also detected at frequencies of $10^{-6}$-$10^{-8}$ (Harris, 1991; Davies and Adams, 1994).

The involvement of the cell wall in the acquisition of nisin resistance by *Listeria monocytogenes* F6861 and its nisin-resistant mutant was investigated. Results indicated that without a cell wall, the acquired nisin resistance of the mutant was lost (Davies *et al.*, 1996). The same researchers showed that cell surface hydrophobicity was related with nisin sensitivity; the wild type strain being more hydrophobic than its mutant. Variation in the phospholipid concentration and changes in that fatty acid composition of the cytoplasmic membranes have been reported to be factors of differentiation between nisin-sensitive and nisin-resistant strains. These tend to confer lower membrane fluidity in resistant strains making it more difficult for nisin to insert and form pores (Ming and Daeschel, 1995).

It has also been reported that nisin's antimicrobial effect is sometimes prevented by a nisin degrading enzyme (dehydroalanine reductase), nisinase. Nisinase has been reported in *Lactobacillus plantarum*, *L. lactis* susp. *lactis*, *L. lactis* subsp. *cremoris*, *Enterococcus faecalis*, *Bacillus cereus* and *Streptococcus salivarius* subsp. *thermophilus* (Harris *et al.*, 1991). Nisinase isolated from *Bacillus cereus* and *Streptococcus salivarius* subsp. *thermophilus* has been partially purified.
Nisin effectiveness is concentration dependent, in terms of both the amount of nisin added and the number of spores or vegetative cells that need to be inhibited. Its action against vegetative bacteria can be bacteriostatic or bactericidal depending on the nisin concentration, bacterial population size, physiological state and conditions of growth. Davies and Delves-Broughton (1999) mention that bactericidal effects are enhanced under optimal growth conditions and when the bacteria are in an energized state. In contrast, bacteriostatic effects are enhanced when nisin forms part of a multi-preservation system when conditions are not optimal and other inhibitory factors are present.

1.3.6 Measuring Nisin Potency

Since 1970 the WHO Committee on Biological Standardization has established as an international nisin reference an International Unit (IU), which was defined as 0.001 mg of this preparation. Nisin is usually expressed as levels of pure nisin (μg/ml or μg/g) and multiplication by 40 converts these values to IU or to equivalent levels of the commercial concentrate Nisaplin®. Thus, 1 μg/g nisin is equivalent to 40 IU/g pure nisin or 40 mg/kg Nisaplin®.

Bioassay techniques are the most common methods used for measuring nisin potency in food products, particularly the horizontal agar diffusion method, which was first developed by Tramer and Fowler (1964). In this method, nisin is extracted from the product by treatment with acid, and a set of controls and standards are included to ensure that the inhibition zones are the result of nisin antimicrobial activity against M. luteus and not due to other antimicrobial factors associated with the food. The size of zones of inhibition is linearly related to the logarithm of nisin at concentrations between 0.03 and 0.5 mg/L.

Additionally, a number of other methods are used for measuring nisin potency, but their application is mainly product dependent. Such methods include the nisin-linked immunosorbent assay (ELISA), the reasurin method, ATP bioluminometry and reverse phase HPLC.
1.3.7 Nisin Applications

Nisin can be regarded as non-toxic when consumed orally and has proven safety as a food preservative (Delves-Broughton, 1990). The susceptibility of nisin to enzymatic degradation could be considered as an advantage for its use in food, as it is quickly digested and cannot have an effect on the intestinal flora or be absorbed into the bloodstream (Heinemann and Williams, 1966). In 1969, nisin was approved for use as an antimicrobial by the Joint Food and Agriculture Organisiation/World Health Organisation Committee on Food Additives, and in 1998 the US Food and Drug Administration declared nisin as GRAS (Generally Recognised As Safe) for use in cheese and processed cheese products (Davies and Delves-Broughton, 1999). Currently, nisin is approved in over 80 countries (labelled E 234), including the USA, the UK and China.

Due its wide antimicrobial activity, nisin is suitable for use in a wide range of food products, including chilled or ambient stored foods (Table 1.1). Typical examples are canned products stored at ambient temperatures in warm climates or products, particularly chilled foods, that have been stored under temperature abuse conditions (Thomas et al., 2000) and it can be partly used as substitute for harsh preservation methods (e.g. thermal processing).

Nisin can be produced in situ by Lactococcus lactis subsp. lactis. Adding the organism into the food can be beneficial in various ways, not only because the gradual release of nisin over time results in reduced storage and processing losses, but because the competitive exclusion of other organisms poses an additional preservative effect (McMullen and Stiles, 1996). On the other hand, undesirable organoleptic changes in the food product due to the addition of the culture and the fact that L. lactis as a mesophile cannot grow at low temperatures, are serious constraints on its use in chilled products.

Nisin as the commercial product Nisaplin® is best added as an aqueous solution, usually to the liquid portion of a product during its processing. It can also be added as a powder, but in this case it is essential to ensure uniform dispersal throughout the food matrix. In principal, the addition of nisin takes place at the last practical stage before heat processing, and the addition
Table 1.1 Examples of nisin application in foods.

<table>
<thead>
<tr>
<th>Type of food</th>
<th>Nisaplin® addition level (mg/kg or litre)*</th>
<th>Typical target organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Processed cheese</td>
<td>100-600</td>
<td><em>Bacillus</em> spp.</td>
</tr>
<tr>
<td>Milk and milk products</td>
<td>10-50</td>
<td><em>Clostridium</em> spp.</td>
</tr>
<tr>
<td>Pasteurised chilled dairy desserts</td>
<td>75-200</td>
<td><em>Bacillus</em> spp. including <em>B. sporothermodurans</em></td>
</tr>
<tr>
<td>Liquid egg</td>
<td>50-200</td>
<td><em>Bacillus</em> spp.</td>
</tr>
<tr>
<td>Pasteurised soups</td>
<td>100-250</td>
<td><em>Bacillus</em> spp.</td>
</tr>
<tr>
<td>Crumpets</td>
<td>150-200</td>
<td><em>Bacillus cereus</em></td>
</tr>
<tr>
<td>Fruit juice</td>
<td>30-60</td>
<td><em>Alicyclobacillus acidoterrestris</em></td>
</tr>
<tr>
<td>Canned food</td>
<td>100-200</td>
<td><em>Bacillus stearothermophilus</em></td>
</tr>
<tr>
<td>Dressings and sauces</td>
<td>50-200</td>
<td><em>Clostridium thermosaccharolyticum</em>, <em>Cl. botulinum</em></td>
</tr>
<tr>
<td>Mascapone cheese</td>
<td>&lt;400</td>
<td><em>Lactic acid bacteria</em>, <em>Brochothrix thermosphacta</em>, <em>Listeria monocytogenes</em></td>
</tr>
<tr>
<td>Meat products such as</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bologna, frankfurter</td>
<td>200-400</td>
<td></td>
</tr>
<tr>
<td>sausages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ricotta cheese</td>
<td>100-200</td>
<td></td>
</tr>
<tr>
<td>Beer, wine and spirits production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pitching yeast wash</td>
<td>1000-1500</td>
<td></td>
</tr>
<tr>
<td>Reduced pasteurisation</td>
<td>10-50</td>
<td></td>
</tr>
<tr>
<td>During fermentation</td>
<td>25-100</td>
<td></td>
</tr>
<tr>
<td>Post-fermentation</td>
<td>10-50</td>
<td></td>
</tr>
</tbody>
</table>


*1 mg Nisaplin® is equivalent to 1000 IU

level depends on the type of food, its heat process, pH, storage conditions and the required shelf-life (Thomas et al., 2000). Nisin can also be used as a spray or dip for surface decontamination, and some attention has been given to its application either alone
or in combination with other antimicrobial agents in packaging films and edible casings (Daeschel et al., 1992; Cutter and Siragusa, 1996; Ming et al., 1997; Padgett et al., 1998).

1.4 MEAT AND MEAT PRODUCTS

1.4.1 Meat as a food system

Meat has always played a significant role in the human diet. Despite moral or religious restrictions within particular consumer groups, it still remains very popular today, due to its desirable texture and flavour characteristics, but also because of the undisputable high biological value of the meat proteins (Adams and Moss, 1995).

Table 1.2 Composition of lean muscle tissue of meat animals (%)

<table>
<thead>
<tr>
<th>Species</th>
<th>Water</th>
<th>Protein</th>
<th>Lipid</th>
<th>Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef</td>
<td>70-73</td>
<td>20-22</td>
<td>4.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Chicken</td>
<td>70-73</td>
<td>20-23</td>
<td>4.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Lamb</td>
<td>73</td>
<td>20</td>
<td>5.6</td>
<td>1.4</td>
</tr>
<tr>
<td>Pork</td>
<td>68-70</td>
<td>19-20</td>
<td>9.11</td>
<td>1.4</td>
</tr>
</tbody>
</table>


The composition of lean meat is relatively constant over a wide range of animals (Table 1.2). Variation is most marked in the lipid content. Meat is considered as a high protein food. Of the total nitrogen content of the muscle, approximately 95% accounts for proteins and only app. 5% for smaller peptides, amino acids and other compounds. The biological value of meat is 0.75 (human milk = 1.0, wheat protein = 0.50) and the net protein utilization is 80 (egg = 100, wheat flour = 52) (Varnam and Sutherland, 1995).

Meat also contains relatively high lipid content. Despite the fact that it is a significant source of energy, especially for persons with increased energy requirements, the high
proportion of cholesterol and saturated fatty acids have been associated with heart
disease, and this is one of the main reasons why a relatively notable segment of
consumers avoids meat. The degree of lipid saturation varies with the type of meat. Beef
has the highest level of saturated fat followed by pork and poultry (Varnam and
Sutherland, 1995).

Water is quantitatively the most important component of meat, accounting for 75% of the
weight. The water content is inversely related to fat content, but remains unaffected by
protein content. Water in meat is associated with muscle tissue and proteins have a
central role in the mechanism of water binding. Water-holding capacity and water-
binding capacity are two parameters of significance in relation to meat texture and
manufacturing properties (Varnam and Sutherland, 1995).

1.4.2 Muscle structure and conditioning

A typical joint of meat from the butchers shop is cut from a number of muscles, each
having its own independent attachment to the skeleton, its own blood supply and its own
nerves. Each muscle is surrounded by a layer of connective tissues consisting almost
entirely of the protein collagen. Structurally, the contractile units of the muscle are the
muscle fibres. These are exceptionally elongated, thin, multinucleate cells bound together
in bundles by connective tissue. Each muscle fibre is surrounded by a cell membrane, the
sarcolemma, within which are contained the myofibrils, complexes of the two major
muscle proteins, myosin and actin, surrounded by the sarcoplasm (Adams and Moss,
1995).

In addition to its high water content and large proportion of protein-originated
components and lipids, meat also contains carbohydrates. Glycogen is a polymer of
glucose held in the liver and muscles as an energy store. In live organisms, glycogen is
broken down via glycolytic and respiratory pathways, to provide energy. On the death of
the animal there is still a demand for energy. In this case, the interruption of respiration
and the continuation of glycolysis result in the conversion of glycogen to lactic acid. The
accumulation of lactic acid and the consequent drop of the pH in the muscle will continue
until the glucoletic enzymes are inactivated by the low pH developed. The pH value of a
typical mammalian muscle in a post-mortem condition is usually 5.2-5.5, provided that there are adequate glycogen reserves.

Lipids are involved in many quality traits of meat. They affect the nutritional value, sensory properties and technological properties. Lipid breakdown begins immediately after the death of the animal, and involves both lipolysis and oxidation. Within muscle lipids, phospholipids comprise 0.5% to 1% of the wet weight of the muscle, and are thought to be the most reactive components. They contain high proportion of long-chained polyunsaturated fatty acids which promote their sensitivity to oxidation, and their amphiphilic structure enables them to interact with water-soluble pro-oxidant agents such as iron or oxygen radicals. In that sense, phospholipids participate in hydrolysis, oxidation and Maillard reactions (Varnam and Sutherland, 1995).

1.4.3 Microbiology of fresh meat

Animal skin and the gastrointestinal tract carry the largest microbial flora, as the internal organs and muscles are protected by the animal’s immune system. The numbers and variation of the microorganisms colonizing the specific sites reflect both the animal’s indigenous microflora and its environment. Contamination of the previously sterile parts of the carcass is possible during various steps in slaughter and butchering. The initial counts are low and range between $10^2$ and $10^4$ when these procedures take place under reasonable hygiene standards. Meat is most commonly stored under chill temperatures aerobically, or in vacuum or modified-atmosphere packing.

Aerobic conditions in combination with low temperatures ($\sim 5^\circ$C) encourage the growth of psychrotrophic aerobes, which rapidly become the dominant spoilage microflora, including genera such as *Pseudomonas*, *Acinetobacter*, and *Psychrobacter*. *Pseudomonas* has been considered to be the specific spoilage microorganism in high-protein foods stored at low temperatures due to its ability to metabolize glucose to gluconate and 2-oxo-gluconate. These two acids accumulate extracellularly and are further metabolized by *Pseudomonas* but not by other competing bacteria after glucose depletion. *Acinetobacter*, and *Psychrobacter* are not able to utilize glucose and probably retain energy through oxidation of amino or organic acids.
A minor component of the spoilage flora includes psychrotrophic Enterobacteriaceae, lactic acid bacteria and the Gram-positive *Brochothrix thermosphacta*. The latter seems to be more important on meats that have higher proportions of fat (like lamb and pork) and thus are of higher pH. Temperatures in excess of 6°C and pH values above 6.5 are thought to promote growth of *Brochothrix thermosphacta*, placing it as a strong competitor for *Pseudomonas*. Enterobacteriaceae cannot compete effectively at low temperatures, but even at slightly elevated temperature conditions which favour their growth, *Pseudomonas* remains the dominant flora.

Off-odours generally appear when counts reach approximately $10^7$ CFU/cm$^2$ and slime is visible when growth is about $10^8$ CFU/cm$^2$. Fruity and putrid odours of meat during spoilage are due to the formation of ethyl esters and sulphur compounds by *Pseudomonas*. Other spoilage metabolites include ammonia, amines, branched chain ester residues, acetoin and diacetyl.

The spoilage pattern in vacuum-packed meat differs significantly from aerobic storage with dominant microflora consisting mainly of lactic acid bacteria. In certain cases there may also be significant growth of *Brochothrix thermosphacta, Shewanella putrefaciens* and Enterobacteriaceae. More recently, psychrotrophic species of *Clostridium* have been recognized as an important potential problem. The rapid depletion of oxygen along with the gradual increase in the concentration of CO$_2$ (app. 20%) restrict the growth of pseudomonads.

Low temperatures, low O$_2$ tensions and their tolerance of CO$_2$ favour lactic acid bacteria growth. Their ascendancy has been attributed to higher affinity to glucose, production of lactic acid and partly to bacteriocin production. *Br. theromosphacta* and *Sh. putrefaciens* are unable to grow at pH values below 6 at low temperatures and low concentration of oxygen. Hence, their presence as part of the flora is possible only when pH conditions are favourably altered. Similarly, members of the Enterobacteriaceae can be detected in vacuum-packed meat when pH and temperature (>5°C) are elevated.

In vacuum-packed fresh meat the main spoilage metabolites include, mainly, L- and D-lactic acids along with acetic, isobutanoic and isopentanoic acids. The presence of the specific compounds and their proportion may vary with the type of lactic acid bacteria.
metabolism (homofermentative or heterofermentative). In higher pH meats, where the presence of \textit{Br. theromosphacta} and \textit{Sh. putrefaciens} is more pronounced, sulphur compounds are also important.

A similar microbial flora is observed in modified atmospheres containing elevated levels of both CO$_2$ and oxygen. Heterofermentative lactic acid bacteria (\textit{Leuconostoc}) can be more numerous due to the stimulatory effect of oxygen, and under certain circumstances Enterobacteriaceae and pseudomonads can be more prevalent. These bacteria along with \textit{Brochothrix thermosphacta} can also be present at higher numbers when prior to packing, conditioning has taken place under aerobic conditions. The spoilage of meat packed under modified atmospheres follows pattern similar to that of vacuum-packed meat involving souring.

Meat animals are known sources of zoonotic pathogens. The extent to which meat can be the source of pathogenic bacteria depends on the degree of contamination at slaughter and further operations, as well as conditions of storage. \textit{Salmonella} is one of the most common pathogens found in raw meat (especially chicken) followed by \textit{Campylobacter}. \textit{Verocytotoxin-producing E. coli} (VTEC) has also been associated with raw beef, and appears to be present in numerous types of meat at retail level (Varnam and Sutherland, 1995).

1.4.4 Microbiology of meat products

1.4.4.1 Uncooked, comminuted and reformed meat products

Uncooked comminuted meat products can be sources of contamination with pathogens and are of poorer overall microbiological quality than whole meat, due to extensive use of ingredients of inferior quality. Meat products of this type are usually stored at temperatures below 4°C to obtain higher shelf lives. Metabisulphite, used as preserving compound in British fresh sausages is active against Gram-negative bacteria (especially \textit{Pseudomonas}), favouring growth of \textit{Br. thermosphacta}, other Gram-positive bacteria and
yeasts. In general, the type and the level of the spoilage bacterial population can differ significantly, depending on the composition of the product, the conditions of handling and storage as well as to the use of preservatives.

Comminuted beef products have been reported to be contaminated by different pathogenic microorganisms, including VTEC, *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Campylobacter*. *Salmonella* has also been found in these products and has been reported to have a higher incidence in fresh pork sausages (Pearson and Dutson, 1987; Varnam and Sutherland, 1995).

**1.4.4.2 Cured meats**

The microbiological stability of cured meats varies considerably according to the technology used, the levels of NaCl and curing agents (nitrate and nitrite, ascorbic acid, polyphosphates and sugars) and storage conditions.

Uncooked sides or joints (Wiltshire bacon) can be spoiled by species of *Micrococcus*, but halophilic *Vibrio* may be of considerable importance. Their presence is indicative of growth having occurred as a consequence of poor temperature control or high humidity during maturation. Enterobacteriaceae and lactic acid bacteria can be found in smaller numbers. The dominant spoilage flora of pre-packed (in vacuum or modified atmospheres) uncooked cured meats stored at refrigeration temperatures consists mainly of lactic acid bacteria (*Lactobacillus*, *Carnobacterium*), particularly in products with low pH and low NaCl content. *Vibrio* and members of the Enterobacteriaceae can occasionally cause spoilage, the populations of the latter arising at excessively high temperatures. Dry-cured hams are stable products with long storage life, occasionally spoiled by the presence of yeasts and moulds externally.

Cooked cured meats are usually retailed as vacuum or modified atmosphere pre-packs. The spoilage microflora typically derives from post-process contamination, consisting, similarly to the uncooked cured products, of *Lactobacillus* and *Carnobacterium* species. Inadequate thermal processing of canned cured hams may result in spoilage by *Lactobacillus* or *Enterococcus* species. Occasionally, even sufficiently processed
products may contain *Clostridium* and *Bacillus* endospores, but outgrowth occurs only in situations of temperature abuse.

Incidence of food-poisoning associated with pathogens in cured meats is considerably low. Nevertheless, the *Salmonella* and Clostridial species have been isolated from vacuum-packed bacon and dry-cured meats. Cooked products of this type have been reported to be vehicles of numerous pathogens, such as *Staph. aureus*, *Salmonella*, *L. monocytogenes*, *Yersinia enterocolitica*, etc (Gardner, 1983; Roberts and Jarvis, 1983; Varnam and Sutherland, 1995).

1.4.4.3 Cooked meat and cooked meat products

Cooked meat products are thought to be stable, when critical processing stages are carried out properly. Changes occurring during cooking may affect microbial growth. For instance Maillard products have been reported to have antimicrobial effects. The use of numerous ingredients added for technological reasons, such as herbs and spices (rosemary, garlic etc.), have been found to be active against bacteria, but at the same time constitute sources of contaminant bacterial endospores. In general, for cooked meats containing insignificant levels of added inhibitors, refrigeration is of prime importance for the control of microorganisms. In cases of elevated storage temperatures (over 7°C), psychrotrophic strains of *Bacillus* and *Clostridium* can be potential sources of spoilage. Marginal underprocessing may result in survival of *Enterococcus* and heat-resistant strains of *Lactobacillus* in cooked meats and sausages. *Pseudomonas* and other Gram-negative oxidase-positive bacteria may constitute common spoilage problem for ready-meals and for unpackaged cooked meats or those packed in air-permeable films.

Food poisoning resulting from insufficient heat processing, is a continuing problem with these products. Salmonellosis outbreaks have been described as the most frequent cases of food-poisoning. Nevertheless, *L. monocytogenes* and *Staph. aureus* have been isolated from such products, indicative of post cooking contamination, inadequate cooking or poor storage temperature control. Low levels of enterotoxin in vacuum or gas-packed products have been attributed to suppression of its production due to low oxygen tensions.
Outbreaks in cooked sausages, pies and puddings have also been associated with *Cl. perfringens* (Varnam and Sutherland, 1995).

### 1.4.4.4 Fermented sausages

Fermented sausages are products of high stability, as a result of the combination of low $a_w$ level and moisture content, reduced pH value, the presence of organic acids and high levels of NaCl and NaNO₂. In large-diameter sausages, the low redox potential further inhibits growth of aerobic bacteria. Moulds and yeasts are normally the only microorganisms capable of growth on fermented sausages and in some occasions species of *Bacillus* and *Micrococcus* have been isolated. Despite the relative freedom from growth of spoilage bacteria, fermented sausages, as raw meat products are considered sources of pathogenic bacteria. *Staph. aureus* has been identified as a major problem in their production because of its relatively resistance at high levels of NaCl and nitrites and due to delayed fermentation onset. Other pathogens isolated from *salami* products, include *L. monocytogenes*, *Salmonella* and VTEC (Varnam and Sutherland, 1995).

### 1.5 NISIN EFFICACY IN MEAT AND MEAT PRODUCTS

The use of nisin in meat systems as a partial replacement of nitrites and to reduce thermal processing has been widely investigated. However, nisin demonstrates variable effectiveness in meat products, thus organisms that show nisin sensitivity *in vitro* are less sensitive when growing in meat. For instance, outgrowth of a resistant *Cl. botulinum* strain was inhibited by 50 μg nisin/ml in laboratory medium, but not by a higher nisin concentration (125 μg/ml) in cooked meat medium (Scott and Taylor, 1981). In most studies investigating nisin efficacy in meat systems, findings vary significantly, and this may be due to the numerous variables that can affect the activity of nisin. The use of different preservation hurdles, like pH, NaCl, vacuum-packaging and low storage temperatures are factors that determine the nature of the microflora and thus the target microorganism for nisin (Rayman *et al*., 1983; Taylor *et al*., 1984; Bell and De Lacy, 1985; Fowler and Gasson, 1991).
The poor activity of nisin in meat has been attributed to factors, such as nisin binding to meat protein, uneven mixing, interference of meat phospholipids with nisin activity and heat lability of nisin in neutral pH conditions (Davies et al., 1999). The effect of different meat components and additives is summarised in Table 1.3.

Nisin binding to proteins has been confirmed by Coventry et al., (1995). They hypothesized that loss of antimicrobial activity was due to nisin reaction with protein sulphhydryl groups, when nisin-containing meat was pasteurized for the manufacture of luncheon corned beef. This study confirms the work of Rayman et al. (1983) in pork meat, who suggested that heating is likely to increase either the strength of nisin binding or the amount of nisin bound. Additionally, strong adsorption of nisin on to muscle proteins, resulting in poor recovery of the polypeptide from meat and meat products, was observed by Bell and De Lacy (1986) and Aasen et al. (2003). The fact that recovery of nisin was reduced even when adsorption capacity was exceeded (all meat adsorption sites occupied) led to the assumption that the excess nisin molecules polymerise with the molecules which are already bound to the meat surface ("super adsorption") (Bell and De Lacy, 1986). This has two main implications; firstly due to polymerization a significant proportion of nisin becomes unavailable for biological action and secondly, the amounts of nisin recovered when using the standard bioassay method become variable.

The interference of fat with nisin activity and recovery has also been investigated in a number of studies, but the results are contradictory. Bell and DeLacy (1986) found that nisin recovery increased with increasing fat content, but the level of recovery decreased for high nisin concentration. It was assumed that the observed results were the combination of two parallel effects; the phenomenon of “super adsorption" to adsorption sites and secondly, fat was physically blocking the adsorption sites. Davies et al. (1999) demonstrated that fat had no effect on the retention or loss of nisin in vacuum-packed Bologna-type sausage, as only approximately 50% of the initial nisin levels were lost by the end of an 80-day storage period irrespective of the fat content. Furthermore, experiments on high and low fat foods showed that fat content has no effect on nisin recovery in solid meats. However, it may have in the case of liquid food and ground meat, where fat and water are mixed, and nisin is likely to adhere to the interface between them (Aasen et al., 2003). The latter is in accordance with previous study confirming the emulsifying properties of nisin in high concentrations (Jaber et al., 2000).
Inhibition of nisin activity by fat has been reported in dairy products. Jones (1974) showed that fat in whole milk inhibited the activity of nisin against *Staphylococcus aureus*. Moreover, Jung *et al.*, (1992) demonstrated that the sensitivity of *Listeria monocytogenes* to nisin in milk was strongly dependent on milk fat concentration. The interference of fat with nisin activity could be similar to known interactions with phospholipid components of the microorganisms' cell membrane.

Enzymatic degradation was investigated by Murray and Richard (1997). Experiments on raw and cooked ground pork meat demonstrated reduced recovery of nisin activity in raw meat in comparison with heated samples, suggesting protease action against nisin. This action is enhanced by homogenisation or grounding of the meat, as they contribute to intracellular protease release (Aasen *et al.*, 2003). Rose *et al.* (1999) developed and used a MALDI-TOF Mass Spectrometry method (Rose *et al.*, 1999) suggested that nisin interacts with glutathione (GSH). Considering that glutathione is an abundant thiol compound in animal tissues (<5mM) and that sulphydryl groups are a known target of nisin it is likely that nisin could react with glutathione (Rose *et al.*, 2002). Comparing raw and heated meat samples, it was assumed that the nisin-GSH reaction was enzyme-mediated, as a mass difference of 307 Da, corresponding to a nisin-GSH adduct formation was observed only in raw meat. Further research carried out by the same group, inducing an *in-vitro* reaction between nisin and glutathione with or without the presence of the enzyme glutathione S-transferase (GST), confirmed the above hypothesis.

Last but not least, pH is another factor that has been studied. Nisin is more stable at low pH (Davies *et al.*, 1998) and the pH of meat and its products is thought to be an additional parameter, restricting the satisfactory use of nisin. This was supported by Rayman *et al.* (1983), who found that nisin was ineffective against *Clostridium botulinum* spores in pork slurries at pH 6, but was effective when the pH was decreased to 5.1.
1.5.1 Nisin activity in raw meat

Nisin has been investigated as a surface treatment for beef and chicken carcass disinfection and on packaged raw meat. At refrigeration temperatures, *L. monocytogenes* strains are able to grow on packaged raw meat stored aerobically and under vacuum conditions (Hudson and Mott, 1993; Hudson et al., 1994). Treatment of packaged steak with a nisin-sorbate combination resulted in significant reduction in the counts of *L. monocytogenes* on beef stored in vacuum and modified atmospheres at refrigeration temperatures (Avery and Buncic, 1997). Cutter and Siragusa (1994, 1996b) showed a significant reduction in the total viable counts, as well as in populations of *L. innocua* and *Br. thermosphacta* on raw beef meat surfaces stored aerobically and in vacuum packs when they incorporated nisin in a pilot scale model carcass washer. On meats artificially inoculated with *L. monocytogenes* and *Staph. aureus*, bacterial growth was delayed for at least one day at room temperature. In a similar experiment at 5°C, growth of *Listeria* was delayed for approximately two weeks and growth of *Staphylococcus* did not occur at all (Chung et al., 1989).

Combination of nisin with other antimicrobial agents and preservation technologies can enhance nisin protection against Gram-positive bacteria and extend nisin activity against Gram-negative bacteria as well. In an investigation of replacing SO₂ with organic acids and nisin in raw pork sausages, Scannell et al., (1997) found that a combination of sodium lactate and nisin was effective in reducing the total bacterial counts of the product, whereas the combination of lactate and the polypeptide protected against *Salmonella* species and *Staph. aureus*. Jarvis and Burke (1976) found that 10 μg nisin/g alone or in combination with 1000 ppm sorbic acid and 2.4% (w/v) polyphosphate were able to delay spoilage of raw British fresh sausages. Polylactic acid (2%), and lactic acid (2%) used on their own or combined with nisin (5 μg/ml) as dipping solutions for fresh beef stored at 4°C under vacuum conditions, were effective in reducing *Pseudomonas* populations and Enterobacteriaceae by 0.7-2.6 log CFU/g respectively (Ariyapitipun et al., 1999). The combination of lactic acid with nisin (10 μg/ml) exhibited the highest reduction in *L. monocytogenes* counts, with less than 1.5 log CFU/g remaining after 42 days of storage (Ariyapitipun et al., 2000).
Table 1.3 Effect of intrinsic and extrinsic factors on the inhibitory action of nisin

<table>
<thead>
<tr>
<th>Function</th>
<th>Component or ingredient</th>
<th>Antagonistic</th>
<th>Synergistic</th>
<th>No effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major food</td>
<td>Starches</td>
<td></td>
<td></td>
<td>X</td>
<td>Pirttijarvi et al. (2001)</td>
</tr>
<tr>
<td>components</td>
<td>Fats/lipids</td>
<td>X</td>
<td></td>
<td></td>
<td>Jung et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>Proteins</td>
<td>X</td>
<td></td>
<td></td>
<td>DeVuyst and Vandamme (1994)</td>
</tr>
<tr>
<td></td>
<td>Glutathione</td>
<td>X</td>
<td></td>
<td></td>
<td>Rose et al. (1999)</td>
</tr>
<tr>
<td>Binders</td>
<td>Phosphates, sodium chloride</td>
<td></td>
<td>X</td>
<td></td>
<td>Jarvis and Burke (1976); Stevens et al. (1992);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Davies et al. (1999); Pawar et al. (2000)</td>
</tr>
<tr>
<td>Carriers</td>
<td>Calcium alginate and agar films</td>
<td></td>
<td>X</td>
<td></td>
<td>Cutter and Siragusa (1997); Natrajam and Sheldon (2000a)</td>
</tr>
<tr>
<td>Chelators</td>
<td>EDTA, citric acid</td>
<td></td>
<td>X</td>
<td></td>
<td>Stevens et al. (1992); Shelef et al. (1995); Boziaris and Adams (1999)</td>
</tr>
<tr>
<td>Emulsifiers</td>
<td>Mono- and diglycerides, glyceride acid esters</td>
<td></td>
<td>X</td>
<td></td>
<td>Henning et al. (1986)</td>
</tr>
<tr>
<td></td>
<td>phospholipids</td>
<td></td>
<td></td>
<td></td>
<td>Bell and De Lacy (1987)</td>
</tr>
<tr>
<td></td>
<td>Monolaurin</td>
<td></td>
<td></td>
<td>X</td>
<td>Thomas et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>Sucrose esters of fatty acids</td>
<td></td>
<td></td>
<td></td>
<td>Yamazaki et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>Diglycerol fatty acid esters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Carcass disinfection using nisin and a chelating agent was achieved in vitro (Cutter and Siragusa 1995a), but similar results were not seen when the same combination solution was used in meat (Cutter and Siragusa, 1995b). However, an optimum treatment of nisin (100 µg/mg), EDTA, citric acid and Tween 20 was described by Shefet et al., (1995) for use on chicken drumsticks to inhibit S. typhimurium and extend shelf life. Zhang and Mustapha (1999) reduced L. monocytogenes populations by approximately 1-2 log CFU/ml in vacuum-packed beef cubes which had been previously immersed in nisin (125 µg/ml) solutions, alone or supplemented with 20 mM EDTA. A slight reduction of E. coli O157:H7 also occurred using the same combinations. In another study, chicken drummettes were soaked for 30 min in nisin solutions containing 25, 50, or 100 µg Nisaplin/ml and 20 or 50 mM EDTA. These treatments resulted in a 2.6 log CFU/ml mean reduction in total aerobic counts per ml of drummettes rinse water after storage at 4°C (Cosby et al., 1999).

Nisin alone (5mg/ml) as Nisaplin® and in combination with 3000 pfu/ml listeriophage significantly reduced populations of L. monocytogenes (2.5 log CFU/ml) in broth and buffer but similar results were not seen in vacuum-packed beef cubes stored at 4°C over 28 days (Dykes and Moorhead, 2002). Vignolo et al., (2000) showed that a two-way and three-way combination of nisin with lactococin and enterocin reduced L. monocytogenes counts in lean beef slurry by 5 to 6 logs CFU/g and over 8 logs CFU/g respectively after 24 hours of exposure. A nisin-producing lactic acid bacterial strain was inoculated into raw beef mince five-to-seven days prior to being processed into frankfurters (Wang et al., 1986). This resulted in an extension of the refrigerated shelf life of vacuum packed frankfurters.

The anti-listerial activity of Nisaplin® 0.4 and 0.8 mg/g in combination with sodium chloride was evaluated in raw buffalo meat mince stored at 37°C and 4°C. The degree of nisin inhibition increased with increasing nisin concentration and lower storage temperature. The presence of sodium chloride (2%) increased the efficacy of nisin at both storage temperatures (Pawar et al., 2000). Nisin antimicrobial activity against Br. thermosphacta, and Carnobacterium sp in culture media, pork juices, and onto lean pork and fat tissue was found to be also enhanced by the addition of lysozyme at varying volumes (Natress et al., 2000).
Combined treatments of high pressure and nisin at different pH values applied to mechanically recovered poultry meat, which is known to have high microbial load, showed that the combination of 100 μg nisin/ml, 350 Mpa and 1% glucono-delta-lactone extended shelf life during a 30 day storage at 2°C (Yuste et al., 1998). A synergistic cidal effect was seen against L. monocytogenes when nisin (2.5 μg/ml) was combined with thermal processing (53°C/3 min), but a reduced effect was observed on turkey carcass skin (Mahadeo and Tahini, 1994).

1.5.2 Nisin activity in cooked meat

The application of nisin as a preservative in cooked products seems to be more promising for several reasons. Since most of these products are fully cooked, the primary bacterial recontamination and growth is limited more to the food surface, thus allowing nisin to be applied to the surface only. Many “further-processed” products are vacuum-packed, creating microenvironment that favours the growth of Gram-positive anaerobic or mesophilic bacteria such as lactic acid bacteria that are more sensitive to nisin than Gram-negative bacteria. Additionally, proteolytic enzymes are inactivated in cooked products.

Nisin has been suggested as a preservative in cooked meats, and as a partial replacement for nitrite in cooked cured meat systems. Outgrowth of Cl. perfringens spores was completely inhibited by a combination of 5 μg/g and 75 ppm of nitrite (Caserio et al., 1979b). Similarly, 1.9-2.5 μg/g nisin in combination with 40 ppm nitrite effectively inhibited outgrowth of spores of Cl. sporogenes for 5 days in meat slurries stored at 37°C (Rayman et al., 1981). In a later study, 12.5 μg/g nisin in combination with 60 ppm nitrite failed to prevent outgrowth of Cl. botulinum spores in pork slurries adjusted to pH 5.8 and held at 25°C. When the pH of the slurry was reduced to pH 5.1, 7.5 μg/g of nisin were able to inhibit completely spore outgrowth (Rayman et al., 1983).

Cured meat products differ significantly in their formulations. Wide variation in the fat content, particle size, salt content, phosphate type, nitrite content, emulsifier, etc is common. Davies et al., (1999) in their study on vacuum-packed bologna-type sausages found nisin to be more effective in sausages formulated with leaner meat and that the type
of the phosphate used as emulsifier was able to affect nisin efficacy in inhibiting lactic acid bacteria (LAB). A longer preservation at 8°C was achieved by using 6.25-25.00 μg/g of nisin along with diphosphate as an emulsifying agent.

Stankiewicz-Berger (1969), found that 100 μg/g Nisaplin® were able to reduce populations of *Lac. viridescens* and *Lac. cellobiosus* in pasteurised (72°C/30 min) frankfurter slants by 7 and 4 log CFU/g, respectively. Nisin (12.5 μg/g) combined with sorbic acid and monolaurin prevented growth of *B. licheniformis* on cured meats (Bell and De Lacy, 1987). A similar amount of nisin when added on its own was able to inhibit the growth of the same spoilage microorganism in cooked luncheon meat stored at 20°C for ten days (Bell and De Lacy, 1986). *L. monocytogenes* was inhibited in liver pate by a combination of nisin, lysozyme and citrate (Teg Steeg, 1993) and in cooked pork tenderloins by surface treatment of 250 μg/ml nisin in combination with modified atmosphere (Fang and Lin, 1994). The combination of nisin with lysozyme and EDTA in cooked ham and Bologna product initially reduced populations of *L. monocytogenes*, *S. typhimurium* and *E. coli* O157:H7 but after four weeks at 8°C they grew back to the same levels as controls (Gill and Holley, 2000).

Aymerich et al (2002) evaluated nisin, sakacin and enterocins, as well as bacteriocin-producing cultures on cooked pork. Nisin (128 AU/g) withstood pasteurisation temperatures, while interactions with meat components had no significant effect on inhibitory activity against *Leuc. carnosum*.

### 1.5.3 Nisin activity in packaging materials

The use of films as antimicrobial delivery systems to reduce undesirable bacteria on foods is not a novel concept but it has recently become an area of expanding interest. Active packaging provides some desired properties other than providing an inert barrier between the product and its environment (Maureillo et al., 2005). Various approaches have been proposed and tried for the use of edible or polymer films to deliver bacteriocins, such as nisin, to food surfaces including muscle foods (Table 1.4). Combining a bacteriocin directly into a polymer material offers several advantages. Firstly, only the necessary
amount of bacteriocin would be used. Secondly, the agent would not be a direct additive to the food, and thirdly, a polymer material made by an edible or biogradable polymer offers environmental advantages (Cutter et al, 2001).
Table 1.4 Examples of nisin applications in non-edible packaging and absorbent packaging pads

<table>
<thead>
<tr>
<th>Food type and storage conditions</th>
<th>Nisin concentration(^a) and co-agents</th>
<th>Bacterial target(s)</th>
<th>Effectiveness</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef, nisin added to films before vacuum-packaging, stored at chill temperatures</td>
<td>Nisin and EDTA in PE and PE-oxide films</td>
<td>Br. thermophila</td>
<td>Reduced by 1.7 to 3.5 log CFU/g over 21 days</td>
<td>Cutter et al. (2001)</td>
</tr>
<tr>
<td>Chicken drumsticks packaged with PVC, LLDPE, and nylon films</td>
<td>100 μg nisin/ml film with 0-7.5 mM EDTA, 3.0-3.1% citric acid, 0-0.5% Tween 80</td>
<td>Salmonella Typhimurium</td>
<td>PVC films most effective, 0.4 to 2.1 log CFU/g reduction</td>
<td>Natrajan and Sheldon (2000a)</td>
</tr>
<tr>
<td>Chicken drumsticks, calcium alginate or agar applied to surface, stored at 4°C</td>
<td>0-1000 μg nisin/ml calcium alginate or agar with 5.0 mM EDTA, 3.0% citric acid, 0.5% Tween 80</td>
<td>Salmonella Typhimurium</td>
<td>1.8 to 4.6 log CFU/g reduction after 96 h</td>
<td>Natrajan and Sheldon (2000b)</td>
</tr>
<tr>
<td>Chicken pieces, commercial absorbent tray pads and pads inoculated with S. Typhimurium, stored at 4°C for 168 h</td>
<td>50 μg/ml nisin, 5 mM EDTA, 3% citric acid, 5% Tween 20 added to pads</td>
<td>Salmonella Typhimurium and mesophilic bacteria</td>
<td>6.3 and 3.1 log CFU/g reductions in mesophilic bacteria and S. Typhimurium, respectively</td>
<td>Sheldon et al. (1996)</td>
</tr>
</tbody>
</table>


\(^a\) 1mg Nisaplin is equivalent to 1000 IU
1.6 AIM OF THE PROJECT

Nisin is the only bacteriocin approved by food legislation in numerous countries and has been successfully used in a wide range of food products of animal or plant origin. Nevertheless, its use in meat and meat products is still limited due to its variable antimicrobial activity, depending on the product composition and storage conditions.

This project investigates separately a number of factors reported to affect unfavourably nisin activity in meat and meat products. Simpler laboratory systems were used in an attempt to explore the mechanism and the extent of interaction of nisin with components found in meat, such as glutathione, proteins and fat. Once mechanisms were clarified, a number of additional treatments or agents were applied in combination with nisin in order to enhance its activity. Experiments were conducted using strains of the *L. monocytogenes*, a significant pathogenic bacterium responsible for food-poisoning outbreaks in meat products.

The specific objectives of this study were as follows:

1. To confirm the formation of the nisin-GSH adduct
2. To show that the reaction is not enzymic
3. To investigate the mechanism of the interaction of glutathione with meat proteins during heat-processing
4. To investigate the mechanism of interaction between glutathione, ascorbic and dehydroascorbic acid, and how this affects nisin efficacy in raw and heat-processed meat
5. To study the combined effect of nisin with different commercial antioxidants
6. To investigate further the possible mechanism of synergy between nisin and antioxidants.
CHAPTER 2
GENERAL MATERIALS AND METHODS
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2.1 ORGANISMS

The organisms used during the project were the following:

*Micrococcus luteus* NCIB 8166, for the nisin bioassay donated by Danisco Beaminster Ltd, Dorset, UK.

*Listeria monocytogenes* NCTC5105, taken from the University of Surrey collection.

*Listeria monocytogenes* Scott A, taken from the University of Surrey collection.

*Listeria monocytogenes* 272, from the collection of Danisco Beaminster Ltd.

*Listeria monocytogenes* 358, from the collection of Danisco Beaminster Ltd.

*Listeria monocytogenes* NCTC12426, from the collection of Danisco Beaminster Ltd.

*Listeria monocytogenes* CRA3930, from the collection of Danisco Beaminster Ltd.

*Bacillus cereus* 2599, from the collection of Danisco Beaminster Ltd.

All the bacteria were stored frozen on beads (Protect; Technical Service Consultants Ltd, Heywood, Lancashire, UK) at -80°C. For resuscitation one bead was added to 10 ml of BHI broth (*L. monocytogenes* strains) or Nutrient broth (*M. luteus*) and incubated at 30°C for 24 hours.

2.2 MICROBIOLOGICAL MEDIA

All the microbiological media were supplied by Oxoid (Basingstoke, Hampshire, UK), unless otherwise stated. For serial decimal dilutions Maximum Recovery Diluent (MRD 0.85% NaCl and 0.1% bacteriological peptone) was used.

2.3 PREPARATION OF NISIN STOCK SOLUTION

Pure nisin has a potency of 4x10^6 IU/ml. A stock solution of nisin was prepared by dissolving 0.01g of purified nisin (Batch no.NP 148-149, Danisco, Beaminster, England) into 50 ml of 0.02 N HCl to give an activity of 10^4 IU/ml. The solution was then sterilised by filtration through a 0.2µm membrane (Filter, Syringe, CA membrane 0.2µUM, sterile, Nalgene, VWR International Ltd, Lutterworth, England).
2.4 NISIN BIOASSAY

The main method applied for the determination and quantification of residual nisin levels was the plate diffusion bioassay procedure, using the nisin-sensitive organism *Micrococcus luteus* NCIB 8166 (Tramer and Fowler, 1964).

2.4.1 Heat/Acid extraction

Representative 10 g portions of each sample were placed into a stomacher bag and 90 ml of 0.02 M HCl were added and homogenised for one minute until the sample was evenly suspended (Seward laboratory blender, Stomacher 400, London, UK). Each homogenised sample was then transferred to 250 ml Duran bottle and the pH was adjusted at pH 2 ± 0.05, using 5 M HCl. The bottles then were placed in a boiling water bath and heated for exactly 10 minutes after the temperature had reached 96°C. Control of temperature was achieved by using a suitable partial immersion thermometer suspended in one of the bottles. Afterwards, the samples were cooled down to room temperature and their pH was adjusted at pH 4 ± 0.05 using 5 M NaOH.

2.4.2 Extraction with water

Similarly to the acid extraction method, samples of 10 g were transferred to a stomacher bag and 90 ml of sterile deionised water were added. The mixture was then homogenised in stomacher lab blender and the suspension was transferred to 250 ml Duran bottles. Sample pH was adjusted at pH 4 ± 0.05 using 5 M HCl.

2.4.3 Sample filtration

Filtration was necessary in those cases where samples were consisted of both aliquid and solid phase, and particulate matter had to be removed before moving to the next steps of sample processing. Approximately 15 ml of the samples were filtered, using 9.0 cm Whatman No 4 filter paper circles into the correspondingly labelled sterile polypropylene universal container. Ideally, the remainder should be filtered into a 1 L Duran bottle using 18.5 cm No 4 filter paper circle, and processed in such a way as to hydrolyse nisin and generate a nisin-free extract which will be used as a diluent for the samples and the
standards. However, because of difficulties in filtration or limited extract volume an Alkalase digested Skimmed-Milk solution (ADSM) was used as diluent.

2.4.4 Preparation of Alkalase Digested Skimmed-Milk (ADSM)

Reconstituted skim milk was prepared by mixing 100 g of skim milk powder with 900 ml of deionised water in glass beaker. The mixture was heated to 62°C, until the powder was evenly suspended into the water. The pH was then adjusted to pH 8.0±0.1 using 5 M NaOH, and 0.65ml/L of alkalase were added. Incubation of one hour at 62°C was required for the enzymic digestion of the milk. A final pH approximately of 6.5 was used as control to determine whether the enzymic digestion was completed successfully or not. Volumes of 50 ml were dispensed in Duran bottles and the contents were autoclaved.

2.4.5 Preparation of the bioassay plate

_Tween 20:_ In a sterile universal container 5ml of Tween 20 (Sigma P-7949, Gillingham, UK) and 5 ml of sterile Maximum Recovery Diluent (MRD) were mixed gently. The mixture was placed in an incubator of 55°C for at least 30 minutes.

_Micrococcus luteus suspension:_ A large number of well isolated _M. luteus_ colonies grown on Iso-Sensitest Agar were transferred and suspended, with the help of a sterile plastic loop, in a testing tube containing sterile MRD. After the suspension was mixed, the tube was placed in a turbidimeter (HACH 2100N, Colorado, USA) and the turbidity of the content was read. Turbidity values between 441 and 458 are acceptable, and correspond to absorbance values ranging from 1.68 to 1.75 at 547 nm. Culture was added or diluted using MRD until acceptable reading was achieved.

_Plate pouring:_ Aseptically 5 ml of the Tween/MRD suspension and the 3.8 ml of the _M.luteus_ suspension were mixed gently with 250 ml of tempered Iso-sensitest Agar. In a flow cabinet the mixture was aseptically poured over the surface of a glass bioassay plate which had been previously sterilised with Industrial Methylated Spirit (IMS). The agar was left to set and the dried plate was placed in the refrigerator for approximately 45 minutes, as chilling facilitates the well-preparation stage.
Nisin standards preparation: A range of five Nisin Reference Standards (20 IU/ml, 10 IU/ml, 5 IU/ml, 2.5 IU/ml and 1.25 IU/ml) was used for the preparation of a five-point standard curve. With reference to this curve the nisin contents of the samples were calculated. Sample extracts were diluted accordingly in order to be included between the highest and lowest values of the standard curve.

Cutting and loading the plate: Using a sterilised borer of 9 mm diameter and allowing 30 mm between adjacent wells and 20 mm between peripheral wells and the edge of the assay plate, 64 to 91 pre-assigned holes were made on the agar according to a random number matrix board which was positioned underneath the assay plate. The agar plugs were then removed with a sterile spatula and the wells were filled with equal volumes of the appropriate samples and five Nisin Reference Standards (20 IU/ml, 10 IU/ml, 5 IU/ml, 2.5 IU/ml and 1.25 IU/ml). Sample extracts were diluted in order to fall in the range of the standard curve. The loaded plate was incubated at 30°C for 18 hours.

Reading and calculating nisin concentration: At the end of the incubation period, the diameter of inhibition zones formed around the wells was measured using digital calipers (CamLab Ltd, Cambridge, UK). All samples and standards were tested in quadruplicate, and the nisin concentration was calculated from the mean inhibition zone produced by each extract or standard by reference to the five-level standard curve.

2.5 pH MEASUREMENT

All pH measurements were performed using an Orion 410A pHmeter (USA).
The antimicrobial activity of nisin as measured with the horizontal agar diffusion method against *M. luteus*.

**Picture 2.1** The antimicrobial activity of nisin as measured with the horizontal agar diffusion method against *M. luteus.*
CHAPTER 3

INTERACTIONS OF NISIN WITH GLUTATHIONE IN A MODEL PROTEIN SYSTEM AND MEAT
CHAPTER 3

INTERACTIONS OF NISIN WITH GLUTATHIONE IN A MODEL PROTEIN SYSTEM AND MEAT

The work in this chapter has been accepted for publication under the title "Interactions of nisin with glutathione in a model protein system and meat" in the Journal of Food Protection

3.1 INTRODUCTION

Nisin is a broad spectrum bacteriocin produced by strains of the food grade lactic-acid bacterium *Lactococcus lactis* subsp. *lactis*. Although used successfully as a preservative in numerous food systems, nisin demonstrates variable effectiveness in meat products, where organisms that show nisin sensitivity *in vitro* are found to be less sensitive when growing in meat. A number of possible reasons for this have been identified. These have included the nature of the spoilage microflora, and preservation parameters (Fowler and Gasson, 1991), nisin binding to meat proteins (Rayman et al., 1983; Bell and De Lacy, 1986; Coventry et al., 1995; Davies and Delves-Broughton, 1999; Aasen et al., 2003), degradation by meat proteases (Murray and Richard, 1997; Aasen et al., 2003), uneven mixing (Davies and Delves-Broughton, 1999), adsorption by fat (Bell and deLacy, 1986; Coventry et al., 1995; Davies et al., 1999; Jaber et al., 2000; Aasen et al., 2003) and pH conditions (Rayman et al., 1983; Davies and Delves-Broughton, 1999).

Glutathione (GSH) (γ-glutamyl-cysteinyl-glycine) is a 307 Da thiol-containing tripeptide. It is found in mammalian cells, plants and microorganisms, and at levels of 156 – 627 nmol/g wet weight in beef, chicken and pork (Wierzbicka et al., 1989), but not in dairy products. It is a strong reducing agent important for cellular defence, especially against free radicals and hydroperoxides. The free sulfhydryl group of GSH has been proposed as the binding site for nisin in fresh meat (Rose et al., 1999). Its reaction with the dehydro
amino acid residues of nisin yields GSH S-conjugates and this has been found to be partially mediated by a glutathione S-transferase (GST) which constitutes about 0.3% of the total soluble protein in bovine muscle (Rose et al., 2002).

Here we report an investigation, which partially supports this observation but indicates that the reaction is non-enzymatic and provides an alternative explanation for the observed enhancement of nisin activity in cooked meats. Therefore the objectives of this chapter are:

a. To show that the formation of the nisin-GSH adduct is not catalysed by GST
b. To investigate the mechanism of the interaction of glutathione with meat proteins during heat-processing.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and media

The organisms used for this study were *Micrococcus luteus* NCIB 8166 and *Listeria monocytogenes* NCTC5105. All bacteria were stored frozen on beads (Protect; Technical Service Consultants Ltd, Heywood, Lancashire, UK) at -80°C. For resuscitation one bead was added 10 ml of BHI broth (*L. monocytogenes*) or Nutrient broth (*M. luteus*) and incubated at 30°C for 24 hours.

3.2.2 Preparation of stock solutions

A stock solution of nisin was prepared as described in the General Materials and Methods (Chapter 2).

A stock solution of glutathione was prepared by dissolving 3.04 g of GSH lyophilised powder (Sigma G6529, Gillingham, UK) into 20 ml of sterile 50 mM phosphate buffer (pH 6.5) to give a concentration of 500 mM. The pH of the solution was then adjusted to 6.5 using NaOH and sterilised by filtration through a 0.2µm membrane (Filter, Syringe, CA membrane 0.2µM, sterile, Nalgene, VWR International Ltd, Lutterworth, England).
Glutathione S-transferase (Sigma G6511, Gillingham, UK) has an activity of 50-100 units/mg of protein and a molecular weight of approximately 40 kDa. A stock solution of 1 mM was prepared by dissolving 40 mg of the enzyme to 1 ml of sterile 50 mM phosphate buffer pH 6.5. Its activity was confirmed using the supplier's protocol.

3.2.3 Measurement of nisin activity

Nisin activity was measured using the horizontal agar diffusion method which is described in detail in the General Materials and Methods (Chapter 2).

3.2.4 Nisin activity in meat

Lean minced beef was purchased locally and was divided into two sachets of 500 g. Each was dosed with 500 IU nisin/g by adding 25 ml of the nisin stock solution and the mixture was blended manually to ensure that nisin was distributed as uniformly as possible. One was immediately transferred to a water bath of 72-73° C temperature. The temperature was monitored using a digital thermometer placed in the middle of a sachet. Once the temperature had reached 70° C it was heated for another two minutes. Samples of 50 g of each portion were transferred to sterilized sachets, vacuum packed and stored at 8° C. Initial extractions of both raw and heated samples were conducted immediately after their preparation. The remainder of two portions was then sampled periodically for nisin extraction. Sample extracts were stored at -18° C before their nisin content was measured.

3.2.5 Nisin extraction

Nisin was extracted from all the samples using either the heat/acid procedure or water extraction. Both are described in detail in the Sections 2.4.1 and 2.4.2 in Chapter 2 (General Materials and Methods).
3.2.6 Nisin activity in meat extract

Lean ground beef meat (320 g) was obtained from a local supermarket (fat content appr. 4.5%), and blended at maximum speed with 5x volume of phosphate buffer (100 mM, pH 7). The blended mixture was then centrifuged (Beckman J2-21M/E, California, USA) at 4 °C for 20 min at 10,000xg and the supernatant filtered through glass wool to remove floating lipids. Volumes of 150 ml of the meat extract were transferred aseptically into 250 ml Duran bottles, and 500 IU nisin/ml added to each as 7.5 ml of the previously prepared stock solution. After the addition of nisin, one of the bottles was transferred to a waterbath at 72-73°C. The temperature was monitored using a digital thermometer placed in the middle of the Duran bottle. Once the temperature had reached 70°C it was heated for another two minutes. Raw and heat-processed meat extracts were stored at 8°C.

3.2.7 Microbial counts

Ten ml samples of raw and heated meat extracts were aseptically removed and blended in a stomacher bag with 90 ml of MRD for 60 seconds using the Stomacher Lab Blender. A decimal serial dilution prepared from this initial dilution was used to prepare replicate PCA plates upon which 0.1 ml of dilutions were spread-plated. Counts were enumerated after incubation at 25°C for 72 hours.

3.2.8 Inactivation of nisin by glutathione

Appropriate amounts of nisin, GSH and GST stock solutions were added to 50 mM phosphate buffer of pH 6.5 in 50 ml sterile polypropylene tubes to produce final concentrations of 2000 IU nisin/ml, 1 μM of GST, and 50, 125 and 250 mM of GSH respectively. Controls containing the same concentrations of nisin and glutathione in the absence of the enzyme were also set up. The experiment was run at two temperatures, 20°C (close to the enzyme’s optimum temperature of 25°C) and at 4°C to represent the chill storage of meat. Samples were taken at time zero, after 12 and 24 hours of storage at 4°C and 20°C respectively.
3.2.9 Glutathione heat stability

This was measured in sterile 50 ml polypropylene tubes containing 50 mM phosphate buffer of pH 6.5. Appropriate amounts of the stock solutions were added to produce final concentrations of 2000 IU nisin/ml, and 50mM and 250 mM of GSH. Samples were heated for 15, 30 and 60 minutes at 72° C, and for 5 min in boiling-water. Additional unheated samples, containing the same nisin and GSH concentrations were prepared as controls. Heat-treated samples and controls were incubated at room temperature (20° C) for a period of 24 hours. Sampling and water extractions of residual nisin were conducted at the beginning and at the end of incubation.

3.2.10 MALDI-TOF MS

All mass spectra were acquired on a linear MALDI-TOF MS equipped with pulsed ion extraction technology (Bruker Proflex™ III; Billerica, Mass., U.S.A.) and a 125 cm flight tube, in a positive ion linear mode with a nitrogen laser (λ=337 nm) for desorption/ionization of the samples and an acceleration voltage of 20 kV. Samples were prepared for MALDI-TOF MS analysis by the method described by Rose and others (1999). A volume of 0.5 μL of the extract was placed on a stainless steel MALDI-TOF probe and allowed to air dry. The probe was then dipped into MilliQ water for 30 seconds to remove water soluble contaminants. Excess water was shaken off and the sample was left to dry. Then 0.5 μL of a saturated solution sinapinic acid (Sigma Chemical, Basingstoke UK.) in a solution containing 2 parts of 0.1% trifluoroacetic acid and 1 part of acetonitrile was added to the sample spot and allowed to air dry. Bovine insulin (MH+ = 5734.557; MH22+ = 2867.782), obtained from Sigma Chemical, was used as a calibrant for external mass calibration. The spectra represent the result of 50 consecutive laser shots.
Diagram 3.1 Schematic representation of MALDI-TOF MS

The ionization method used with this instrument is matrix-assisted laser desorption ionization or MALDI. The type of mass analyzer is a time-of-flight or TOF with reflector capabilities. Initially, samples are diluted appropriately, mixed with the matrix, and then 1 µl of solution is spotted on a plate. Once spots are dried the plate can be inserted into the MALDI-TOF MS for analysis. There are various spotting methods that can be used and a variety of matrices that can be used. Thus, some method development needs to be done, because every protein is unique. The matrices are organic acids, such as alpha-cyano-4-hydroxy cinnamic acid (CHCA) or sinapinic acid (SA). A nitrogen UV laser (337 nm) is used to generate ions. The ions are then accelerated in an electric field (25 kV) to move down the flight tube. In linear mode, the ions travel down a linear flight path and their mass/charge ratio is determined by the time it takes an ion to reach the detector. In reflectron mode, ions are reflected back at slight angle towards a detector (Bruker Proflex™ III; Billerica, Mass., U.S.A).
3.2.11 Inhibition of *L. monocytogenes* by nisin in the presence of BSA and GSH

The ability of nisin to inhibit the growth of *L. monocytogenes* NCTC5105 in 5 mM GSH solutions which had been previously heated in the presence or absence of 10% (w/v) BSA was examined. Briefly, 5 mM of GSH were heated at 72°C for 2 min in the presence or absence of BSA (10% w/v). Nisin (from stock solution) diluted in double strength BHI broth was added after mixtures had cooled down to room temperature to give a final concentration of 50 IU nisin/ml. Test and control samples were incubated at 20°C for 24 hours and then an overnight culture of *L. monocytogenes* was diluted properly in MRD to give a final inoculum of approximately 10^3 CFU/ml. Changes in the population of *L. monocytogenes* were monitored over a period of 40h at 30°C and enumerated on BHI agar.

3.2.12 Measurement of free thiol content in BSA-GSH solution

The content of free thiol groups was measured in heated (72°C for 2 minutes) and non-heated BSA (10% w/v) solutions with 2.5, 1.25 and 0.625 mM GSH. Solutions containing the same concentrations of GSH only, and BSA only were also prepared and treated similarly. Standards of known GSH concentrations were prepared and used as reference for the calculation of the thiol contents of the test solutions. The thiol content of test and standard samples was determined using Ellman's method (Ellman, 1959). Briefly, 3 ml of sample extracts prepared in 80% ethanol were mixed with 2 ml of 100 mM phosphate buffer pH 8 and 5 ml of deionised water. Then 0.2 ml of 5,5'-dithiobis'2-nitrobenzoic acid (DTNB)(Sigma, Gillingham, UK) dissolved in 100 mM phosphate buffer pH 7 was added to 3 ml of the previously prepared mixture in a photometer cuvette. Colour developed within 2 minutes and the absorbance was measured at 412 nm.

3.2.13 Statistical evaluation

For all experiments three independent trials were conducted and measurements were performed in duplicate, unless otherwise stated. Statistical evaluation of results was performed using SPPS package v.11.0 for Windows using ANOVA and Student's t test.
Figure 3.1 Calibration curve for the calculation of GSH concentration in 80% ethanol based on DTNB absorbance measurement at 412 nm. Points represent mean of three replicate experiments tested in triplicate.
3.3 RESULTS

3.3.1 Determination of the fate of nisin in raw and heat-processed lean beef meat

In this first experiment the decrease in residual nisin levels in raw and heat-processed minced meat in vacuum packs during storage at 8°C was measured (Figure 3.2, 3.3).

For raw meat residual nisin released by acid/heat extraction had an initial level of approximately 440 IU/g, representing 88% recovery. However, within the first six hours there was a rapid drop of almost 200 IU/g, to a level of 263 IU/g. After this period, nisin decreased steadily, reaching 112 IU/g at the end of the incubation.

With water extraction only 23% of nisin was recovered immediately after its addition. Within the first 50 h of incubation at 8°C nisin levels decreased steadily and then they stabilised at around 15–20 IU/g until the end of the experiment.

Bound nisin was calculated by subtracting water-extracted nisin values from acid-extracted nisin values. The former represents ‘free’ nisin, thus nisin which is theoretically available for biological action, whereas heat-acid treatment releases the total amount of nisin added to the samples, including nisin bound to food components.

In pasteurised meat, nisin was added before heat processing. The initial value of nisin immediately after heat-treatment was extracted from pasteurised samples, using acid/heat method and had an average initial value of 490 IU/g, showing a 98% recovery. After the second day of storage at 8°C nisin content dropped steadily to a level of 330 IU/g. This was followed by an apparent increase, to fall again to a level of 204 IU/g at the end of storage period.

A water extraction performed immediately after the nisin addition to heated samples resulted in average detectable nisin levels of 53 IU/g. This was estimated at 11% of the original addition levels. During storage at 8°C nisin recovery remained at levels similar
to those initially detected; unlike the nisin recovery obtained using heat/acid extraction. Bound nisin was calculated by subtracting water-extracted nisin values from acid-extracted nisin values.

As a general observation, the rate of total nisin loss during storage is significantly faster in raw meat compared to that in heat-processed samples. After only three hours of storage at 8°C the total nisin recovered from raw meat was at similar levels to those of total nisin detected in heated samples after 23 hours. However, the nisin levels in acid/heat extracted samples fluctuated significantly throughout storage, making interpretation difficult.

The rate of loss of free nisin in pasteurised minced beef meat was significantly slower than that in raw meat. In raw meat samples, more than 50% of the free nisin present initially was lost within the first ten hours of storage (Figure 3.4). After 24 hours nisin levels declined more slowly levelling out at around 20% of the initial concentration. Although heat processing itself reduced the nisin content by about 12%, the heat-processed meat retained almost 80% of the remaining nisin activity after 24 hours of storage and at the end of the fourth day the same samples contained approximately 50% of the initial level (Figure 3.4).
Figure 3.2 Measurement of nisin contents in raw lean beef meat stored at 8°C - Comparison of two extraction methods. Acid/heat-extracted nisin (●), water-extracted nisin (■), and bound nisin (▲). Each point represents the mean ± standard deviation of three replicate samples assayed in quadruplicate.

Figure 3.3 Measurement of nisin contents in heat-processed lean beef meat (70°C, 2 min) stored at 8°C - Comparison of two extraction methods. Acid/heat-extracted nisin (●), water-extracted nisin (■), and bound nisin (▲). Each point represents the mean ± standard deviation of three replicate samples assayed in quadruplicate.
3.3.2 Determination of the fate of nisin in lean beef meat extracts

Interpretation of the last experiment was difficult, as it suffered from technical difficulties, such as uneven mixing of nisin in the food matrix. Thus, the same experiment was repeated in an aqueous meat extract where total (heat/acid extractable) residual nisin was estimated (Figure 3.5).

In a raw meat extract, the nisin detected by bioassay immediately after addition, using heat/acid extraction, was 100%. After the first four hours of storage at 8 °C the content of nisin in raw meat extracts had decreased over 150 IU/ml, to a value of 370 IU/ml. After 12 hours 42% of the nisin detected initially was lost, and only 35% of the original nisin levels was detected at the end of storage.

Heating nisin in the raw meat extract resulted in an initial loss of 12% of the original addition levels. During the first four hours only 10% of total nisin was lost, but subsequent storage resulted in further losses, with nisin activity levelling at 307 IU/ml (70%). At the end of storage time approximately 61% of the initially added nisin has recovered.

Addition of nisin to meat extract after thermal processing prevented any nisin losses due to nisin interactions with components of the meat extract. Nisin detected by acid/heat extraction had an initial concentration of approximately 500 IU/ml. Similar to extracts heated with nisin present, nisin levels were stable over the first seven hours of storage and decreased to below 80% over the next five hours. Nisin content then declined only slightly until the end of storage.

Comparing the three different meat-extract treatments, it was clear that nisin recovery immediately after sample preparation is poorer in heat-processed samples as heating has resulted to an initial loss of 60 IU/ml. On the other hand, detection levels in raw meat-extract samples and in meat extracts that have been heated before nisin addition were 100%.

After four hours of storage at 8° C, there were significant differences in nisin recovery between the raw meat extract and the heated ones (p<0.05). After 12 hours nisin recovery
from raw meat extract was 208 IU/ml (42%), in heated sample it was almost 70% and in pre-heated meat extract 75% of the initial addition. At the end of storage nisin was recovered from raw meat-extract at approximately 35% of the original levels. Better recovery was achieved in heated meat extracts and pre-heated samples.

In addition to the nisin-treated samples, two nisin-free meat extracts, a raw and a heated one were also investigated for microbial growth. Over the first 48 hours of storage total viable counts remained below detection limits for all samples tested. Thereafter, only in the nisin-containing raw meat-extracts and those where no nisin addition was made, microbial numbers increased to approximately 4 and 3 log CFU/ml respectively. (Table 3.1).

Table 3.1 Microbial growth on raw and heated (70°C/2min) nisin dosed and nisin-free meat extracts. Counts are representative of two replicate experiments in duplicate.

<table>
<thead>
<tr>
<th>Hours</th>
<th>Nisin-Free Raw Meat extract</th>
<th>Nisin-Free Heated Meat extract</th>
<th>Raw Meat Extract + Nisin</th>
<th>Heated Meat Extract + Nisin</th>
<th>Pre-Heated Meat Extract + Nisin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>24</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>48</td>
<td>3.38</td>
<td>&lt;2</td>
<td>4.33</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>72</td>
<td>3.78</td>
<td>&lt;2</td>
<td>5.20</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>
Figure 3.4 Loss of ‘water extractable’ nisin content in lean vacuum packed minced beef stored at 8°C. Raw meat (●), heated meat (70°C, 2 min) (■). Each point represents the mean ± standard deviation of three replicate samples assayed in quadruplicate.

Figure 3.5 Recovery of “heat/acid extractable” nisin content in lean beef meat extracts stored at 8°C. Each point represents the mean ± standard deviation of two replicate samples assayed in quadruplicate. Nisin content was not estimated for pre-heated meat extract after 4 hours of storage.
3.3.3 Inactivation of nisin by glutathione

Nisin inactivation by glutathione in the presence or absence of the glutathione S-transferase was evaluated by measuring its antimicrobial activity against *Micrococcus luteus* in the standard bioassay. The results shown clearly confirm a loss of nisin activity in the presence of glutathione (Fig 3.6).

The results also show, that formation of the nisin-glutathione adduct occurs largely through a non-enzyme mediated reaction. At the higher temperature, 20°C, in the presence of 250 and 125 mM of glutathione, formation of the adduct resulted in a complete loss of detectable nisin activity, with less than 1% of the initial nisin activity recovered from both enzymatic and control reactions after 24 hours. Under conditions where residual nisin was detectable there was no significant difference (p> 0.05) between levels in the enzyme treated samples and controls. At 4°C, using three concentrations of glutathione and two sampling times, there was significantly greater loss of nisin in the presence of enzyme (p< 0.05) on only one occasion (250 mM glutathione after 12h) on all other occasions there was no significant difference between the nisin loss with or without enzyme (p> 0.05).

There was also good correlation between the concentration of glutathione and nisin recovery which was more pronounced at 4°C. Increasing levels of glutathione resulted in reduced nisin activity after a period of 24 hours (Fig 3.7).
Figure 3.6 Nisin recovery (%) following reaction with glutathione in the presence or absence of glutathione S-transferase at 4°C (a), and 20°C (b), for 12 and 24h in phosphate buffer. Each point represents the mean of three replicate samples assayed in quadruplicate.
Figure 3.7 Correlation between glutathione concentration and nisin recovery (%) for enzyme free samples incubated at 4°C. Correlation coefficient was found to be -0.97, indicating that nisin activity loss in the presence of glutathione is dose-dependent.
3.3.4 Glutathione heat stability

Lower loss of nisin in the cooked meat is not a result of degradation of glutathione by heat. Glutathione solutions that had been heated at 72°C for one hour or 100°C for 5 minutes retained the ability to inactivate nisin. With 250 mM glutathione solutions there was no significant difference in the loss of nisin when incubated with heated or unheated glutathione solutions for 24 h (p> 0.05). The mean loss in nisin activity was 97%. At 50 mM glutathione, the difference between heated and unheated solutions was again not significant with a mean loss of nisin activity loss of 74% (Table 3.2).

3.3.5 MALDI-TOF MS

MALDI-TOF Mass spectrometry was used to detect the products of the reaction of nisin with glutathione (250 and 50 mM) in heated and control solutions (5, 50 and 250 mM) at the end of a 24-hour post-treatment incubation period. Figures 3.8, 3.9 and 3.10 illustrate the results of the experiment. The mass spectra of the heat-treated and untreated glutathione (250 mM) solutions were compared. In both cases, MALDI-TOF analysis revealed a single peak of approximately 3,966 Da (≈ 3,353+ 614 Da) which correlates to the addition of 2 molecules of glutathione on one nisin molecule. The original nisin peak (≈3,352 Da) was not detected (Fig. 3.9a and b). The results obtained for the samples with the lower glutathione concentration also demonstrate the nisin-glutathione adduct formation. For the heated samples three peaks of approximately 3,352 Da, 3,659.688 Da and 3,966.933 Da were detected and were assumed to correspond to nisin, nisin + 1 molecule of GSH (≈3,352 + 307 Da) and nisin + 2 molecules GSH (≈3,352 + 614 Da) (Fig. 3.10a). For the untreated samples only the original peak and a second one approximately of 614 Da greater were detected (Fig 3.10b).
Table 3.2: Nisin loss (%) following reaction with glutathione in heated (72°C/2 min) and untreated solutions after 24 hour of storage at 20°C in phosphate buffer. Each point represents the mean of three replicate samples assayed in quadruplicate.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>250 mM GSH+ Nisin</th>
<th>50mM GSH+ Nisin</th>
<th>Nisin Alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>72°C</td>
<td>0 min</td>
<td>98.30 ± 0.31</td>
<td>73.00 ± 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 min</td>
<td>94.80 ± 1.02</td>
<td>68.10 ± 0.07</td>
<td>14.00 ± 2.60</td>
</tr>
<tr>
<td></td>
<td>30 min</td>
<td>94.90 ± 5.23</td>
<td>63.10 ± 0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 min</td>
<td>96.00 ± 3.27</td>
<td>66.00 ± 0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 min</td>
<td>96.30 ± 0.87</td>
<td>81.65 ± 4.55</td>
<td></td>
</tr>
<tr>
<td>100°C</td>
<td>5 min</td>
<td>96.60 ± 1.49</td>
<td>80.40 ± 4.06</td>
<td>17.9 ± 5.74</td>
</tr>
</tbody>
</table>
Figure 3.8 Formation of the glutathione-nisin adduct using MALDI-TOF MS; nisin alone (a), 5 mM GSH (b), 50 mM GSH (c) and 250 mM GSH (d) in phosphate buffer at 20°C.
Figure 3.9 Formation of the glutathione-nisin adduct, using MALD-TOF MS; 250 mM GSH heat-treated (a), and 250 mM GSH untreated (b).
Figure 3.10 Formation of the glutathione-nisin adduct using MALD-TOF MS; 50 mM GSH heat-treated (a), and 50 mM GSH non treated (b).
3.3.6 Interaction of glutathione with meat proteins during heating

Heating glutathione with the soluble protein bovine serum albumin, BSA, removed the ability of glutathione to reduce nisin activity. Nisin (50 IU/ml) was able to inhibit *Listeria monocytogenes* when it was left for 24 h in a solution of glutathione (5 mM) and BSA (10%) which had been heated at 72°C for 2 minutes. Counts of *L. monocytogenes* remained below detection limit over a 40-hour incubation at 30°C. However when the same level of nisin was left with heated glutathione for 24h, it lost activity and *L. monocytogenes* was able to grow to levels in excess of $10^8$ CFU/ml within 20 h (Figure 3.11).

As shown in Table 3.3, in heated BSA solutions containing different concentrations of glutathione the absorbance of DTNB at 412 nm was decreased in all cases, indicating that glutathione interacts with protein molecules during heat-induced gelation. There was also a slight but insignificant reduction of the free thiol groups in controls containing glutathione or BSA only after heat treatment.

The results from both methods clearly demonstrate that reduced nisin loss in heat-treated meat may be due to glutathione binding on meat proteins during heat processing and not the result of heat inactivation of glutathione S-transferase.
Figure 3.11 Inhibition of *L. monocytogenes* growth in BHI broth by nisin a) after 24 hours in preheated GSH plus BSA (•), b) after 24 hours in preheated GSH (▲), c) after 24 hours in preheated BSA (★), d) 24 hours in BHI (+). Each point represents the mean ± standard deviation of two replicate samples tested in triplicate. Arrows represent undetectable counts.

Table 3.3 Reduction of free thiol content of BSA plus GSH solutions immediately after heat treatment. Each value represents the mean ± standard deviation of three replicate samples determined in triplicate.

<table>
<thead>
<tr>
<th>Sample Composition</th>
<th>Reduction of free thiol content after heat treatment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 mM GSH + 10% BSA</td>
<td>35.22 (± 0.02)</td>
</tr>
<tr>
<td>1.25 mM GSH + 10% BSA</td>
<td>39.67 (± 0.01)</td>
</tr>
<tr>
<td>0.625 mM GSH + 10% BSA</td>
<td>40.20 (± 0.03)</td>
</tr>
<tr>
<td>2.5 mM GSH</td>
<td>5.84 (± 0.04)</td>
</tr>
<tr>
<td>1.25 mM GSH</td>
<td>2.64 (± 0.03)</td>
</tr>
<tr>
<td>0.625 mM GSH</td>
<td>6.6 (± 0.03)</td>
</tr>
<tr>
<td>10% BSA</td>
<td>1.30 (± 0.02)</td>
</tr>
</tbody>
</table>
3.4. DISCUSSION

Retention of bacteriocin activity in food matrices is essential for the inhibition of pathogenic and spoilage bacteria during food production and storage. Due to their nature, bacteriocins are vulnerable to interactions with other food macromolecules, resulting in reduced efficacy.

Results presented here have shown that nisin recovery immediately after its addition to the food matrix was dependent on the extraction method used. Water-extractable nisin was approximately 23% of the initially detected levels compared to 88% for heat/acid extraction. These findings are in accordance with previous studies. Aasen et al. (2003) studied the fate of nisin and sakacin P in three different products (cold-smoked salmon, chicken cold cuts and raw chicken) using three different extraction methods: urea, acetic acid and water. Urea is known to break both hydrophobic and ionic bonds, while pH reduction breaks only the ionic bonds, by neutralising the negative charges at the muscle proteins, preventing binding of the cationic bacteriocin (Aasen et al., 2003). Similar nisin recoveries after urea and acid extractions led to the assumption that ionic interactions between the bacteriocins and the food components dominate. The same authors found that 10-20% nisin was recovered by water extraction compared to 100% recovery with urea extraction. Similar experiments on raw ground pork stored at 5°C, also showed that nisin recovery using hot acid extraction was four times higher than that when cold neutral extraction was applied (Murray and Richard, 1997). Results for pasteurised meat showed a similar pattern of nisin recovery using different extraction methods.

Poor nisin recovery is believed to be caused by strong adsorption of nisin on to meat proteins (Bell and De Lacy, 1986; Coventry et al., 1995; Davies et al., 1999; Aasen et al., 2003). Adsorption decreases with falling pH, but meat must still be boiled at an acid pH to liberate the protein-bound nisin. The present results substantiate this hypothesis as the levels of nisin extracted using water alone were significantly lower compared to the levels obtained after acid/heat extraction. A more rapid drop in total nisin content (heat/acid-extractable) compared to that of free nisin (water-extractable) was seen for both ground meat and meat extracts. This may be due to bound nisin acting as a reservoir replenishing free nisin levels.
Total nisin levels in raw minced meat dropped rapidly particularly within the first six hours of incubation with a rate of approximately 90 IU/ml per three hours. The decrease in total nisin in heat-processed minced meat and meat extracts was lower as recovery was more than 70% and 60% respectively, after 24 hours. Differences in nisin retention between solid meat and liquid systems may be due to conformational differences, e.g., increased binding sites for nisin, resulting in lower amounts of nisin left for biological action.

Low total viable counts in meat extracts revealed that reduction in free nisin in raw samples was not caused by microbial activity. Increased loss of nisin activity in raw meat compared with cooked meat had been taken as evidence of the activity of enzymes naturally present in meat. Murray and Richard (1997) found that nisin activity declined with storage time, with a more rapid loss in the raw meat than heat-treated meat regardless the mode of extraction used; concluding that reduced recovery in raw meat could be due to protease action. Aasen et al., (2003) attributed reduced bacteriocin stability in raw foods to enzymic activity which can be more pronounced in liquid food systems due to the release of intracellular proteases during homogenisation. Indeed, comparison of our results obtained from raw minced meat and meat extracts revealed a more rapid loss of total nisin in the meat extract. Rose et al., (1999) used matrix assisted MALDI-TOF MS to determine the fate of nisin in meat and meat juice after samples were soaked in nisin solutions for one hour and stored overnight at 4° C. Nisin was recovered from cooked meat extract and cooked meat juice, but only nisin bound to a food component was detected in fresh meat extract. No "free" nisin was detected on raw meat after overnight storage at 4° C. The mass increase in nisin of approximately 307 Da in raw meat extract in combination with the loss of activity led the authors to conclude that nisin reacted enzymatically with glutathione, as this phenomenon did not occur in cooked meat. This assumption is also supported by Sipes and Gandolf (1991) who hypothesised that nisin exhibits some features common to known substrates of glutathione S-transferase, including its hydrophobicity and the fact that it contains an electrophilic carbon. Parkenson (1996) postulated that the apparent role of glutathione S-transferase in glutathione conjugation reactions is to increase the rate of the reaction by assisting the deprotonation of GSH to GS−. In vitro experiments involving nisin and glutathione in the presence or absence of glutathione S-transferase claimed to show that the reaction product is the result of a reaction assisted by enzymic activity (Rose et al., 2002).
The work reported here demonstrates that the non-enzymatic reaction accounts for a major part of the conjugation-reaction between nisin and glutathione, which can proceed adequately in the absence of glutathione S-transferase, regardless of the glutathione concentration or temperature.

It was hypothesised and shown that retention of nisin activity in cooked meat was due to loss of free sulfhydryl groups during cooking as a result of the reaction of glutathione with proteins and not the result of enzymic inactivation. Glutathione has been found to be involved in gel formation of proteins (Legomo et al., 1993) by reducing intramolecular disulfide bonds to sulfhydryl groups (Jocelyn, 1972; Haschemeyer and Haschemeyer, 1973). Reduction of disulfide bonds involves conformational changes and subsequent exposure of hydrophobic regions which promotes the interaction of proteins and leads to gel formation through disulfide linkages (Hayakawa and Nakamura, 1986; Li-Chan and Nakai, 1991). The consequent loss of free glutathione in this way will reduce glutathione-nisin adduct formation.

In this study, heating of glutathione with protein was shown to reduce significantly the levels of free sulfhydryl groups indicating that glutathione interacts with proteins. This was confirmed by the inhibitory effect of nisin against L. monocytogenes after it had been left for 24 h in a solution of glutathione and BSA.

This study indicates that reduced nisin loss in heat-treated meat is partly due to glutathione reaction with meat proteins during heat processing and not the result of heat inactivation of glutathione S-transferase.
CHAPTER 4

THE PROTECTIVE EFFECT OF DEHYDROASCORBIC ACID ON NISIN ACTIVITY
CHAPTER 4

THE PROTECTIVE EFFECT OF DEHYDROASCORBIC ACID ON NISIN ACTIVITY

4.1 INTRODUCTION

Nisin can be used as a preservative in meat products such as emulsified sausages (e.g. hot dogs, wiener frankfurters), cooked meat or poultry products (ready-to-eat meals, soups, sauces), raw meat marinades, meat injection systems, ground raw meats, etc. In these meat products nisin is normally used for the control of Gram-positive bacteria such as species of Bacillus and Clostridium, Listeria monocytogenes and spoilage lactic acid bacteria. Although nisin has been used successfully in certain meat systems (Rayman et al., 1981; Chung et al., 1989; Fang and Lin, 1995; Beuchat et al., 1997; Budu-Amoaka et al., 1999; Davies et al., 1999) its activity in those systems is often less than expected from comparative test results in laboratory media. Among other factors, glutathione has been proposed to reduce nisin activity in raw meat due to the formation of an inactive nisin-glutathione adduct (Rose et al., 1999; 2002; 2003). Higher levels of added nisin are recovered from heat-processed meat as a result of glutathione interactions with meat proteins during heat-processing (Stergiou et al., 2006).

In bread making, glutathione is also involved in exchange reactions between neighbouring sulphhydryl groups of gluten proteins resulting in dough more resistant to mixing. Quantities of approximately 10 - 15 mg/kg glutathione are found in flour in the free form (GSH), the oxidized dimer (GSSG) and bound to protein (PSSG). In 1935, it was discovered that small additions of ascorbic acid (AA) caused increased dough strength and bread volume.

The effect of ascorbic acid takes place in two steps. When the flour, water and other ingredients are mixed, ascorbic acid is oxidised to dehydroascorbic acid (DHAA) by oxygen present in the dough. The reaction is catalysed by an ascorbic acid oxidase, an
enzyme naturally present in flour. The reaction could also be catalysed by heavy metals or heme-containing compounds such as the enzymes peroxidase or catalase (1).

(1) \[ AA + \frac{1}{2} O_2 \rightarrow DHAA + H_2O. \]

The second step involves the conversion of GSH to its disulfide GSSG by the oxidising product, DHAA, by means of a second enzyme (glutathione dehydrogenase) also present in flour (2).

(2) \[ DHAA + 2GSH \rightarrow AA + GSSG \]

The oxidized dimeric form of GSH is inactive in disulfide exchange reactions of dough development (Belitz & Grosch, 1999; Coultate, 2002).

Antioxidants such as ascorbic acid and erythorbate (isoascorbate) are commonly used in meat. Sodium nitrite (used in cured meat products) is a strong oxidant, which can react with endogenous or added reductants such as ascorbic acid or erythorbate to produce nitric oxide. Nitrous acid (HNO₂), the conjugate acid of nitrite, is unstable in solution and decomposes in a reversible fashion.

(3) \[ 3HNO_2 \rightarrow H^+ + NO_3^- + 2NO + H_2O. \]

In solutions nitrous acid can behave as both a reductant and an oxidant, but in meat systems the role is primarily as an oxidant, with oxidizing capacity increased at low pH. In a reaction with ascorbic acid, nitrous acid is reduced to produce the free-radical product NO, while ascorbic acid is simultaneously oxidized to yield DHAA.

(4) \[ AA + 2HNO_2 \rightarrow DHAA + 2NO + 2H_2O \]

Meat also contains heme-containing compounds that could affect conversion of AA to DHAA (Varnam & Sutherland, 1995).

The aim of this study was to investigate the possibility of dehydroascorbic acid acting in the same way in meat as it does in dough, thereby protecting nisin. The use of nisin,
ascorbate or erythorbate and sodium nitrite in meat systems is known (Davies et al. 1999). Therefore, the oxidation of ascorbic acid by sodium nitrite was also assessed as a mean of generating dehydroascorbic acid.

4.2 MATERIALS AND METHODS

4.2.1 Cultures

For the purposes of this study *Listeria monocytogenes* (Scott A, NCTC5105, 272, 358, NCTC12426 and CRA3930) and *Micrococcus luteus* were used. After resuscitation suspensions were subcultured on a weekly basis on BHI agar (*L. monocytogenes*) and Iso-Sensitse agar (*M. luteus*), and they were maintained at 4°C. All strains were grown at 30°C for 15-18 hours to reach stationary phase, prior to their use as inocula.

4.2.2 Nisin

Nisin was provided as *Nisaplin®* (Aplin & Barrett Ltd) which has an activity of $1 \times 10^6$ IU/g. A nisin stock solution of 10,000 IU/ml was prepared by dissolving 0.1g of Nisaplin in 0.02M HCl and making the volume up to 10ml. This was centrifuged at 3000rpm for 5 minutes, from which the supernatant was retained and filter sterilised through a 0.2µm membrane (Filter, Syringe, CA membrane 0.2µM, sterile, Nalgene, VWR International Ltd, Lutterworth, England). The stock solution was then diluted in sterile distilled water to give the required range of concentrations.

4.2.3 Chemicals

Appropriate amounts of reduced glutathione (Sigma Chemicals, Gillingham, UK) were dissolved in phosphate buffer (50 mM, pH 6.5) to give 500 mM and 50 mM solutions. The pH of the stock solutions was readjusted with NaOH at 6.5 ± 0.1, and then filter sterilised through a 0.2µm membrane (Nalgene, VWR International Ltd, Lutterworth, England). DHAA (Sigma Chemicals, Gillingham, UK) was dissolved in 50 mM
phosphate buffer pH 6.5 whilst being stirred and heated at ~90°C. The final solution was 50 mM, with pH adjusted to 6.5 ± 0.1. This was filter sterilised through a 0.2 μm membrane (Nalgene, VWR International Ltd, Lutterworth, England).

4.2.4 Minimum Inhibitory Concentrations (MIC) for nisin

*L. monocytogenes* MICs were performed in BHI broth, and *M. luteus* was tested in nutrient broth. A range of nisin concentrations (50 IU/ml-1500 IU/ml) were set up by adding 1 ml of the prepared nisin solutions to the broths. The overnight cultures of the three bacterial strains were serially diluted in MRD from $10^{-1}$ to $10^{-7}$ from which 0.1 ml aliquots of the $10^{-4}$ solutions were used to inoculate the MICs. All MICs had a final volume of 10 ml and were performed in triplicate and with nisin-free controls. All test tubes were incubated at 30°C and observed for turbidity after 24 and 48 hours. The MIC was determined by the nisin concentration at which bacterial growth is fully inhibited. Enumeration of initial inoculums was performed by triplicate spread plating of the serial dilutions on either BHI agar (*L. monocytogenes*) or Iso-Sensitest agar (*M. luteus*), which were incubated at 30°C and counted for colonies after 24 hours.

4.2.5 MICs for nisin and glutathione

Given the sensitivity of *M. luteus*, only the *L. monocytogenes* NCTC5105 and Scott A strains were tested from this stage of the experiment onwards. The MICs were set up as previously described but with 1 ml of the BHI broth replaced with 1 ml of the GSH stock solutions. Two different concentrations of GSH were tested; 5 mM and 50 mM, and prior to inoculation, the BHI broths containing nisin and GSH, were incubated at 20°C for 24 h, to allow the reaction between nisin and GSH. Again all MICs had nisin and GSH free controls and were tested in triplicate. Incubation conditions were as described above.

4.2.6 MICs for nisin, glutathione and dehydroascorbic acid

MICs were set up similarly to previous experiments. Glutathione was used in a concentration of 5 mM, and DHAA was tested at concentrations of 2.5 mM and 5 mM. The GSH and DHAA were added to the broths first and allowed a 45-minute reaction period
prior to the addition of nisin. Combinations of nisin with GSH and/or DHAA were stored at 20°C for 24 h prior to inoculation with the test strains. Controls and enumerations were all run simultaneously and incubations were as before. The pH of the tests containing all compounds alone and in combinations was also measured.

4.2.7 *In situ* production of DHAA

Dehydroascorbic acid (DHAA) would be too expensive to use in meat and is not allowed as a food additive. It was hypothesised that sodium nitrite (SN) would oxidise AA to DHAA, which would then react with GSH to prevent nisin inactivation. Stock solutions of AA (Sigma, Gillingham, UK) and SN (Sigma, Gillingham, UK) were prepared by dissolving 0.176 and 0.138 g respectively into 10 ml of 50 mM phosphate buffer pH 6.5. According to the stoichiometry of the reaction (see section 4.1), 10 mM of AA were mixed with 20 mM SN to generate 10 mM DHAA. Control solutions of ascorbic acid (10 mM) and sodium nitrite (20 mM) were also prepared.

4.2.8 HPLC experiments

Solutions of ascorbic acid and sodium nitrite were prepared as previously described. The compounds were left to react for one hour at room temperature and the product of their reaction was further reduced using DTT in ratio 1:8. Reaction time was one hour in the dark at 20°C (Gokmen *et al.*, 2000). Excess DTT was used to ensure that all the produced DHAA would be reduced back to AA. The reaction product, its reduced product, as well as six AA standards were prepared and diluted appropriately to fall in the range of the detection limits of the HPLC instrument. Analysis of AA was achieved using High-Performance Liquid Chromatography (HPLC) with electrochemical detection. All injections were made by the autosampler. The method used was adopted by Washco *et al.* (1989) with slight modifications. Briefly, the mobile phase consisted of 0.05 M sodium phosphate, 0.05 M sodium acetate and 189 mM tetraoctylammonium bromide in 30/70 methanol/water (v/v), at pH 4.8. The stationary phase was a Waters Spherisorb 5mm ODS2 4.6x150mm analytical column (Waters, Ireland). For detection an LC4C amperometric detector was used. Operating voltage was set at 0.5 V.
4.2.9 Spectrophotometry experiments

Oxidation of ascorbic acid by sodium nitrite was monitored in a Vis-UV spectrophotometer at 265 nm (λ max for AA at pH 6.0-6.8) at room temperature. The reduction of DHAA by GSH was also investigated using the same method. Solutions of AA (10 mM), GSH (20 mM), NaN0₂ (20 mM) and DHAA (5 mM) were freshly prepared in sterile phosphate buffer (50 mM, pH 6.5) and were properly diluted in the same buffer to generate final working conditions consisted of 0.5 and 1 mM AA, 2 mM GSH, 2 mM NaN0₂ and 1 or 0.5 mM DHAA.

The level of free GSH was also measured using the method described by Ellman (1959). Briefly, 3 ml of sample extracts prepared in 50 mM phosphate buffer pH 6.5 were mixed with 2 ml of 100 mM phosphate buffer pH 8 and 5 ml of deionised water. Then 0.2 ml of 5, 5'-dithiobis'2-nitrobenzoic acid (DTNB)(Sigma, Gillingham, UK) dissolved in 100 mM phosphate buffer pH 7 was added to 3 ml of the previously prepared mixture in a photometer cuvette. Colour developed within 2 minutes and the absorbance was measured at 412 nm.

Standards of known GSH and AA concentrations were prepared and used as reference for the calculation of the AA and GHS contents of the test solutions (Fig 4.1, 4.2).
Figure 4.1 Calibration curve for the calculation of AA concentration in phosphate buffer, (50 mM, pH 6.5) based on direct absorbance measurement at 265 nm. Points represent mean of three replicate experiments tested in triplicate.

\[ y = 0.5493x + 0.0313 \]
\[ R^2 = 0.9992 \]

Figure 4.2 Calibration curve for the calculation of GSH concentration in phosphate buffer, (50 mM, pH 6.5) based on DTNB absorbance measurement at 412 nm. Points represent mean of three replicate experiments tested in triplicate.

\[ y = 1.6369x + 0.1612 \]
\[ R^2 = 0.994 \]
4.2.10 Bactericidal experiments using commercial and in situ DHAA in BHI broth

Solutions of commercial dehydroascorbic acid (DHAA), ascorbic acid (AA), sodium nitrite (SN) and their mixture (in situ produced DHAA) were prepared as described above. Appropriate volumes of each compound solution were added singly or in combinations to BHI broth to obtain final working conditions consisting of 200 IU nisin /ml, 5 mM GSH, 2.5 and 5 mM commercial DHAA, 5 and 10 mM in situ produced DHAA, 5 and 10 mM AA and 20 mM SN.

The volumes of all broths were 9.9 ml and were held for 24 hours at 20°C prior to inoculation. Following this, a culture of *Listeria monocytogenes* grown overnight was added to generate initial suspension of approximately $10^8$ CFU/ml. Sampling was performed at the following intervals; 0, 5, 15, 30 and 60 minutes. At these times 1ml aliquot of each broth was used to set up a dilution series in MRD a selection of which was then spread plated in duplicate onto BHI agar. All plates were incubated at 30°C for 24 hours, and then counted for colonies of *L. monocytogenes*. These counts were then converted into log CFU/ml for each stage of the experiment in order to be plotted as survivor curves.

4.2.11 Bactericidal experiments using commercial and in situ DHAA in meat extract

To demonstrate the protective effect of commercial and in situ produced DHAA in a model meat system, meat extract was prepared and used for a similar assessment of nisin activity in the presence/absence of GSH +/- DHAA.

Lean ground beef meat was obtained from a local supermarket (fat content appr. 4.5%), and blended at maximum speed with 5x volume of phosphate buffer (100 mM, pH 7). The blended mixture was then centrifuged (Beckman J2-21M/E, California, USA) at 4°C for 20 min at 10,000xg and the supernatant filtered through glass wool to remove floating lipids. Appropriate volumes of the nisin stock solution were added in the meat extract to generate final nisin levels ranging from 0-300 IU nisin/ml. Following this, meat extracts were inoculated with *L. monocytogenes* NCTC5105, which had been grown overnight at 30°C in BHI broth. Bacterial growth was monitored over 48 h and counts were enumerated on BHI agar.
In further experiments investigating the loss of nisin activity in neat extracts and its restoration in the presence of 5 mM DHAA the experimental protocol was changed, and nisin-containing tests with or without DHAA were incubated at 20 °C for 24 h prior to the addition of the inocula. The same procedure was followed when in situ produced DHAA (10 mM) was used alternatively. Single additions or in combination with nisin of AA (10 mM) and SN (20 mM) were also made. PH was measured immediately after mixing and at the end of pre-incubation at 20°C for 24 h, to ensure that pH would not have an effect on *Listeria* growth.

Growth of *Listeria monocytogenes* NCTC5105 was monitored for 48 h at 30°C. At sampling times 1ml aliquot of each test was used to set up a dilution series in MRD. A selection of these dilutions was then spread plated in duplicate onto BHI agar. All plates were incubated at 30°C for 24 hours, and then counted for colonies of *L. monocytogenes*. These counts were then converted into log CFU/ml for each stage of the experiment in order to be plotted as survivor curves.

### 4.2.12 Bactericidal experiments using commercial and *in situ* DHAA in meat slurry

Solutions of commercial dehydroascorbic acid, ascorbic acid, sodium nitrite and their mixture (*in situ* produced DHAA) were prepared as described above. Minced beef meat (fat content < 4.5%) was purchased from local supermarket and 100 g of this were homogenised with equal quantity of sterile deionised water in a stomacher bag for 60 sec. The homogenate was then divided into portions of 18 g and single or combined additions of the compounds were made, to generate final working conditions consisting of 100 IU nisin/g, 5 mM commercial DHAA, 10 mM *in situ* produced DHAA, 10 mM AA and 20 mM SN. All mixtures were then left for 12 hours at 4°C, prior to inoculation with *L. monocytogenes* NCTC5105 at approximately 10⁷ CFU/g.

A second set of the same tests was also prepared and used for pH measurement and determination of the total viable microbial content (on BHI and MRS agar) before and after the pre-incubation period. The bactericidal effect of nisin and its combination with DHAA, AA, SN and *in situ* produced DHAA was monitored over a period of two hours. Samples were taken at 5, 15, 30, 60, 90 and 120 min. At sampling times 1ml aliquot of
each test was used to set up a dilution series in MRD. A selection of these dilutions was then spread plated in duplicate onto *Listeria* Selective Agar (Oxford formulation). All plates were incubated at 30°C for 24 hours, and then counted for colonies of *L. monocytogenes*. These counts were then converted into log CFU/ml for each stage of the experiment in order to be plotted as survivor curves.

### 4.2.13 Investigation of the nisin protective effect of DHAA in cooked meat slurry

Raw meat slurry was prepared as before (pH 5.78). Additions were made as appropriate of nisin (100 IU/ml) and dehydro-ascorbic acid (2.5 mM). The pH of the meat was checked and re-adjusted. The meat was left at ambient temperature for 30 minutes, then pasteurised at a core temperature of 90°C for 10 minutes. After cooling the meat tests were inoculated with $10^2$ CFU/g of a *Listeria monocytogenes* cocktail (strains 272, 358, NCTC12426, CRA3930). The meat tests were stored at 8°C and sampled at regular intervals. Counts were performed on *Listeria* Selective Agar (Oxoid, Basingstoke, UK).

### 4.2.14 Investigation of the nisin protective effect of AA and nitrite in cooked meat slurry

Raw meat slurry was prepared as above (pH 6.33). Additions, singly or in combination, were made to the meat of AA (5 mM), sodium nitrite (10 mM) and nisin (100 IU/g). The meat was held at ambient temperature for 30 minutes, then pasteurised at a core temperature of 90°C for 10 minutes, cooled and inoculated with a *Listeria monocytogenes* strain cocktail (as above). The meat samples were incubated at 8°C and regularly analysed by viable count enumeration on *Listeria* Selective Agar (Oxoid, Basingstoke, UK) as described above.

### 4.2.15 Data analysis

For all experiments two independent trials were conducted and measurements were performed in duplicate, unless otherwise stated. Statistical evaluation of results was performed using SPPS package v.11.0 for Windows using ANOVA and Student's t test.
4.3 RESULTS

4.3.1 Minimum Inhibitory Concentrations (MIC) for nisin

The minimum inhibitory concentration in each experiment was judged to be the concentration at which no growth (turbidity) was visible. Triplicate tubes were used for this to be ascertained more accurately. The first trials set up were designed to find the MIC for each strain of *L. monocytogenes* and the *M. luteus* against nisin alone. The *M. luteus* was very sensitive to nisin, and as such it was not possible to ascertain an MIC (25 IU/ml was the lowest nisin concentration tested) in the first two trials, therefore all further work focused on the *L. monocytogenes* strains. The NCTC 5105 strain was tested seven times, in triplicate resulting in 21 MIC results. The Scott A strain was tested five times, to give a total of 15 MICs. Figures 4.3 and 4.4 depict the 48 h MIC values recorded. From these results, the mode nisin MICs for *L. monocytogenes* NCTC 5105 and Scott A are 200 IU/ml and 250 IU/ml respectively.

4.3.2 Nisin and glutathione MICs

Two different concentrations of glutathione were initially tested in combination with nisin against *L. monocytogenes* NCTC 5105. Based on the results in Chapter 3, nisin was incubated with GSH at 20°C for 24 hours prior to inoculation to allow formation of the nisin-GSH adduct and inactivation of nisin. With both 5mM GSH and 50mM GSH the MICs were elevated after 24 hours and increased again after 48 hours incubation. The 50mM GSH experiments resulted in bacterial growth across the full range of nisin concentrations, and as such an end point MIC could not be determined, >1500 IU/ml. Therefore in the later experiments, only 5mM GSH was used. The MICs (n = 15) with 5mM GSH were raised to between 500 IU/ml and 1500 IU/ml after 24 hours and generally increased to the next higher nisin concentration after 48 hours. For the Scott A strain a similar pattern was seen. After 24 hours the MIC was typically 750 IU/ml, rising to >1000 IU/ml after 48 hours (n = 9). This demonstrates reduced efficacy of nisin against *L. monocytogenes* in the presence of 5mM GSH (Figure 4.5).
Figure 4.3 *L. monocytogenes* NCTC 5105 Nisin MICs after 48 hours. (No of replicates=21).

![Bar chart showing Nisin MICs for *L. monocytogenes* NCTC 5105 after 48 hours.](image)

Figure 4.4 *L. monocytogenes* Scott A Nisin MICs after 48 hours (No of replicates=15)

![Bar chart showing Nisin MICs for *L. monocytogenes* Scott A after 48 hours.](image)
4.3.3 Nisin, GSH and dehydroascorbic acid MICs

The next stage involved the addition of DHAA, at 2.5mM and 5mM. The GSH concentration remained constant at 5mM. Nisin alone and nisin plus 5mM GSH MICs were run simultaneously and the results for NCTC5105 strain were 200 IU/ml and 1500 IU/ml respectively (mode MIC values). When dehydroascorbic acid was added along with nisin and GSH the mode MICs generated after 48 hours was 100 IU/ml for both 2.5 and 5 mM of DHAA (Fig 4.6a, b).

Scott A was also tested and after 48 hours the MIC with 2.5mM DHAA was 250 IU/ml (Fig. 4.7a). With 5mM DHAA the MIC after 48 hours was 100 IU/ml in most of the tubes tested (Fig. 4.7b). This indicates a lowering of the nisin MIC when DHAA is added.

The addition of GSH to the MIC system clearly affects the bacteriostatic effect of nisin on both strains, causing the MIC to rise considerably. The addition of DHAA into the system not only resolves this problem, but can actually lower the MIC. Figure 4.8 illustrates the mode MIC results for each strain of *L. monocytogenes*, in each MIC system tested.

![Figure 4.5 Nisin MIC test results following pre-incubation at 20°C for 24 hours in the presence of 5 mM GSH against *L. monocytogenes* NCTC5105 and *L. monocytogenes* Scott A, after 48 h at 30 °C in BHI broth.](image-url)
Figure 4.6 MICs after 48 hours for *L. monocytogenes* NCTC5105 following pre-incubation at 20°C for 24 hours in the presence of nisin, 5 mM GSH, (a) 2.5 mM DHAA and (b) 5 mM DHAA.

Figure 4.7 MICs after 48 hours for *L. monocytogenes* Scott A following pre-incubation at 20°C for 24 hours in the presence of nisin, 5 mM GSH, (a) 2.5 mM DHAA and (b) 5 mM DHAA.
Figure 4.8 Summary of mode MIC results for *L. monocytogenes* NCTC5105 and Scott A after 48 hours for all the model systems tested.

4.3.4 Bactericidal effect of nisin and/or commercial DHAA in the presence of GSH in BHI broth

Previous experiments demonstrated the bacteriostatic effects of nisin, its antagonism by GSH and resolution of this using DHAA. A series of experiments were performed to look at the bactericidal effects of this system on *L. monocytogenes* NCTC 5105 by enumerating survivors periodically over sixty minutes (Figure 4.9). Where glutathione was added it was always pre-incubated for 24 h at 20°C before the bacteria were added to allow reaction with nisin.

The results demonstrated the rapid action of nisin alone against *L. monocytogenes*, producing a mean reduction of two logs in survivors after 5 min. Overall, within the 60 minute period, viable counts were reduced by approximately 3.5 log cycles. The addition of 5 mM GSH led to a more stable bacterial count over the 60 minutes, showing that nisin activity was completely negated by pre-incubation with glutathione (Table 4.1).
The bactericidal activity of nisin in the presence of GSH was not only restored, but was enhanced when GSH was mixed with DHAA for 45 min prior to nisin addition and pre-incubation (for 24 h at 20°C). Surviving populations after 5 min were 5.05 and 4.65 log CFU/ml for 2.5 and 5 mM of DHAA respectively, significantly lower than with nisin alone (p<0.05). After 60 min, the bactericidal effect of nisin was significantly more pronounced for tests containing 5 mM DHAA with approximately 5 log cycles reduction (p<0.05). The increased lethality in the presence of DHAA was found to be a dose dependent phenomenon at least after the first 5 min of exposure (p<0.05), with surviving populations being: nisin alone > nisin + 2.5 mM DHAA > nisin + 5 mM DHAA.

The control tests containing GSH (5 mM) and DHAA (5 mM) on their own or combined showed that the strain survived well over the experimental period with no loss of cells. Therefore GSH and DHAA did not have any significant independent effect on the survival of *L. monocytogenes* NCTC 5105.

![Figure 4.9](image)

**Figure 4.9** Effect of nisin (200 IU/ml) on *L. monocytogenes* NCTC5105 in BHI broth following pre-incubation for 24 h at 20°C with GSH (5 mM) +/- DHAA (2.5 and 5 mM). Each point represents the mean value ± standard deviation of two independent samples tested in duplicate.
Table 4.1 Count reduction in *L. monocytogenes* in BHI broth after 5 and 60 min of exposure to 200 IU nisin/ml after pre-incubation for 24 h at 20°C with GSH (5 mM) and/or DHAA (2.5 and 5 mM).

<table>
<thead>
<tr>
<th>Combinations</th>
<th>Counts&lt;sub&gt;0&lt;/sub&gt; (Log CFU/ml)</th>
<th>Counts&lt;sub&gt;5&lt;/sub&gt; (Log CFU/ml)</th>
<th>Counts&lt;sub&gt;60&lt;/sub&gt; (Log CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisin</td>
<td>$7.88 \pm 0.14$</td>
<td>$5.95 \pm 0.35^{a,b}$</td>
<td>$4.42 \pm 0.08^{c}$</td>
</tr>
<tr>
<td>Nisin + GSH</td>
<td>$7.66 \pm 0.11$</td>
<td>$7.92 \pm 0.33$</td>
<td>$7.55 \pm 0.06$</td>
</tr>
<tr>
<td>Nisin + GSH +5 mM DHAA</td>
<td>$7.65 \pm 0.16$</td>
<td>$4.65 \pm 0.23^{a}$</td>
<td>$2.71 \pm 0.71^{c}$</td>
</tr>
<tr>
<td>Nisin + GSH +2.5 mM DHAA</td>
<td>$7.72 \pm 0.16$</td>
<td>$5.05 \pm 0.35^{b}$</td>
<td>$4.17 \pm 0.81$</td>
</tr>
</tbody>
</table>

*a*: Statistical significance between samples (p<0.05) estimated using Student’s t-test  
*b*: Statistical significance between samples (p<0.05) estimated using Student’s t-test  
*c*: Statistical significance between samples (p<0.05) estimated using Student’s t-test

4.3.5 Oxidation of ascorbic acid in the presence of sodium nitrite

Dehydroascorbic acid is not a permitted food preservative. Ascorbic acid and nitrite are permitted however, and are used in many formulated meat products. The possibility that nitrite could oxidise ascorbic acid to produce dehydroascorbic acid (DHAA) was investigated.

A solution of ascorbic acid (10 mM) in phosphate buffer (50 mM, pH 6.5) was mixed with NaNO<sub>2</sub> dissolved in the same buffer (20 mM). The mixture was diluted properly to final working conditions of 0.5 and 1 mM AA and 1 and 2 mM NaNO<sub>2</sub>. Decrease in the absorbance at 265 nm was monitored over 10 min at room temperature. As shown in Figure 4.10 ascorbic acid was completely oxidised over a period of 60 and 90 min for 0.5 and 1 mM of ascorbic acid respectively. The calculated slope was higher when the sodium nitrite concentration was doubled and the time for it to reduce the AA concentration to the half was less than with the 1 mM of sodium nitrite (Table 4.2).
Figure 4.10 Oxidation of 1 mM and 0.5 mM ascorbic acid at room temperature in the presence of sodium nitrite a) 1 mM (*) and b) 2 mM (•) respectively.

Table 4.2 Kinetic parameters of ascorbic acid oxidation in the presence of sodium nitrite

<table>
<thead>
<tr>
<th>Concentration_{NaNO2}</th>
<th>Concentration_{AA}</th>
<th>Slope</th>
<th>T[AA]_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM</td>
<td>0.53 mM</td>
<td>-0.038</td>
<td>25 min</td>
</tr>
<tr>
<td>2 mM</td>
<td>0.98 mM</td>
<td>-0.058</td>
<td>15 min</td>
</tr>
</tbody>
</table>

4.3.6 Detection of *in situ* produced DHAA using HPLC

The product of ascorbic acid oxidation by sodium nitrite was detected using HPLC with electrochemical detection. As shown by chromatogram A (Fig 4.11) ascorbic acid at a concentration of 0.01 mM was eluted from the column after 11 minutes. The ascorbic acid peak was totally eliminated from the chromatogram after treatment with sodium nitrite, indicating that all the ascorbic acid had been oxidised. When the product of the oxidation was reduced using DTT, the ascorbic acid peak re-appeared. The signal this time was weaker and this can be taken as evidence that some of the dehydroascorbic was further degraded to 2,5 diketogulonic acid. This last step of the reaction is irreversible.
Figure 4.11 HPLC elution profiles of ascorbic acid. (a) 0.01 mM of ascorbic acid, (b) 0.01 mM of ascorbic acid oxidised by 0.02 mM of sodium nitrite, c) Reduced DHAA to AA after incubation at 20°C in the dark or 1 hour.
4.3.7 Bactericidal effect of nisin and/or \textit{in situ} produced DHAA in the presence of GSH in BHI broth

The bactericidal effect of nisin in the presence and absence of GSH, ascorbic acid, sodium nitrite and the \textit{in situ} produced dehydroascorbic acid via the oxidation of ascorbic acid by sodium nitrite was also investigated. Where glutathione was added, it was pre-incubated with nisin for 24 h at 20°C to allow the reaction between nisin and glutathione to occur.

As shown in Figure 4.12 the addition of GSH to the broth totally inactivated nisin over 24 hours so that when \textit{Listeria} suspension was added, counts remained at levels similar to those determined initially (7.6 log CFU/ml). In the absence of GSH, nisin produced about 2 log cycles reduction after the first five minutes of exposure and after 60 min survivors were estimated at 3.2 log CFU/ml.

Two levels of \textit{in situ} produced dehydroascorbic acid were tested, using 10 and 5 mM of ascorbic acid. As shown by the results, the presence of the \textit{in situ} produced dehydroascorbic acid inhibited formation of the inactive GSH-nisin adduct, preventing nisin inactivation. The total reduction in \textit{Listeria} counts after 60 min of exposure was 4.3 and 3.0 log CFU/ml for 10 and 5 mM respectively.

The combination of nisin with ascorbic acid in the presence of GSH was also shown to restore completely nisin activity. Surviving populations after one hour were similar to those enumerated for the \textit{in situ} produced dehydroascorbic acid (3.1 log CFU/ml for 10 mM of AA and 4.4 log CFU/ml for 5 mM of AA). However, the bactericidal effect produced after the first five minutes of exposure was more pronounced compared to that of the dehydroascorbic acid and this was observed with both concentrations of ascorbic acid.

Addition of sodium nitrite (20 mM) in the nisin-glutathione system had no effect on nisin activity and the slight reduction (0.7 log CFU/ml) in the counts was presumably due to the high concentration of sodium nitrite used.
As shown in Figure 4.13 the presence of ascorbic acid or dehydroascorbic acid enhanced nisin activity. The combination of nisin with the commercial preparation of dehydroascorbic acid (5 mM) produced approximately a five log cycle reduction. The in situ dehydroascorbic acid also improved nisin activity even though the logarithmic count reduction was less (about 4.3 log CFU/ml) than this seen with the commercial product and with AA alone (about 4.5 log CFU.ml). This was possibly to losses due to further oxidation of DHAA. A similar pattern was seen with the lower concentrations but the reduction in all cases was less, suggesting that the extent of stimulation in the nisin activity is concentration dependent.

When nisin was combined with DHAA (5 mM) and AA (5 mM) in the absence of glutathione the lethal effect was shown to be more pronounced compared to that seen in the presence of glutathione or nisin alone (Fig 4.14).

![Graph](image_url)

**Figure 4.12** Effect of nisin (200 IU/ml) on *L. monocytogenes* NCTC5105 in BHI broth following pre-incubation for 24 h at 20°C in the presence of GSH (5 mM) +/- AA (5 and 10 mM), SN (20 mM) and in situ produced DHAA (5 and 10 mM). Each point represents the mean value ± standard deviation of two independent samples tested in duplicate.
Figure 4.13 Comparative logarithmic reductions in *L. monocytogenes* counts after 60 min of exposure to nisin-containing BHI following pre-incubation for 24 h at 20°C in the presence of GSH (5 mM), DHAA (2.5 and 5 mM), AA (5 and 10 mM), and *in situ* produced DHAA (5 and 10 mM).

Figure 4.14 Effect of nisin (200 IU/ml) on *L. monocytogenes* NCTC5105 in BHI broth following pre-incubation for 24 h at 20°C in the presence of AA (5 mM) and commercial DHAA (5 mM) +/- GSH (5 mM). Nisin (*), Nisin + AA + GSH (*), Nisin + DHAA + GSH (▲). Open symbols in the absence of GSH. Each point represents the mean value ± standard deviation of two independent samples tested in duplicate. Arrows mark undetectable counts (below 10 CFU/ml).
4.3.8 Bactericidal effect of nisin in the presence of DHAA in raw meat extract

To demonstrate the protective effect of dehydroascorbic acid in a liquid system more closely resembling meat, filter sterilised raw meat extract was prepared and used for a similar assessment of nisin activity in the presence/absence of GSH +/- DHAA. Initial tests showed that 100 IU nisin/ml of meat extract were sufficient to inhibit totally the growth of *L. monocytogenes* when the nisin was added immediately before the bacterial suspension (Fig 4.15). When nisin was left in the meat extract for 24 h at 20 °C prior to inoculation, the minimum inhibitory concentration increased to levels higher than 300 IU/ml (the highest test level), indicating that nisin was partly inactivated due to reaction with free GSH and possibly other sulfhydryl groups present in the extract (Fig 4.16). The addition of DHAA to the meat extract prior to nisin addition and the subsequent incubation at 20 °C for 24 h prior to inoculation, restored nisin activity. After 48 hours at optimum growth temperature, survivor numbers remained below detectable levels (<10 CFU/ml). Dehydroascorbic acid used on its own also slightly retarded growth (Fig 4.17).

![Figure 4.15 Minimum Inhibitory concentration of nisin against *L. monocytogenes* NCTC5105 in meat extract. Arrows mark undetectable counts (below 10 CFU/ml). Each point represents the mean value ± standard deviation of two independent experiments tested in duplicate.](image)
Figure 4.16 Minimum Inhibitory concentration of nisin against *L. monocytogenes* NCTC5105 in meat extract after incubation at 20°C for 24 h prior to inoculation. Arrows mark undetectable counts (below 10 log CFU/ml). Each point represents the mean value ± standard deviation of two independent experiments tested in duplicate.

Figure 4.17 Growth of *L. monocytogenes* NCTC5105 at 30°C for 48 h in meat extract, where nisin (100 IU/ml) was pre-incubated with 5mM DHAA for 24 hours at 20°C prior to inoculation. Arrows mark undetectable counts (below 10 CFU/ml). Each point represents the mean value ± standard deviation of two independent samples tested in duplicate.
In further experiments the commercial preparation of dehydroascorbic acid was replaced by *in situ* produced dehydroascorbic acid. The effect of ascorbic acid and sodium nitrite on their own with nisin was also assessed. As shown in Figure 4.18 with nisin which had been previously left in the meat extract for 24 hours survivors recovered after 24 hours and after another 24 hours of incubation at 30°C counts had exceeded 6 log CFU/ml. The presence of sodium nitrite and nisin in the meat extract did not further sensitise cells and the pattern of recovery was similar to that with nisin alone.

Sodium nitrite and ascorbic acid on their own slightly retarded growth but when the latter was combined with nisin it rendered survivors undetectable for over 48 hours. The same was observed in the presence of the *in situ* produced DHAA, indicating that the addition of these compounds probably inactivated glutathione and enhanced the inhibitory effect of nisin.

**Figure 4.18** Growth of *L. monocytogenes* NCTC5105 at 30°C for 48 h in meat extract, where nisin (100 IU/ml) was pre-incubated with 10 mM AA, 20 mM SN and their combination (*in situ* produced DHAA) for 24 hours at 20°C prior to inoculation. Arrows mark undetectable counts (below 10 CFU/ml). Each point represents the mean value ± standard deviation of two independent samples tested in duplicate.
The pH of the mixtures was measured to ensure that improved nisin activity was not the result of lower pH which is known to enhance nisin solubility. In the first trials, where a commercial preparation of dehydroascorbic acid was used the initial pH ranged between 6.83 (for the control) and 6.57 (for the combination of nisin and DHAA), and slightly decreased after 24-hour storage at 20°C prior to the addition of the Listeria suspension (Table 4.3).

When commercial dehydroascorbic acid was replaced with in situ produced dehydroascorbic acid, initial pH ranged between 6.62 (for the control) and 6.15 (for the combination of nisin with ascorbic acid). After 24 hours at 20°C no significant changes were observed in the pH values compared to the initial ones for each combination separately (Table 4.4).

Table 4.3 Listeria growth in meat extract after 48 h at 30 °C. The protective effect of DHAA on nisin stored for 24 h at 20 °C prior to inoculation.

<table>
<thead>
<tr>
<th>Test Combinations</th>
<th>pH_{initial}</th>
<th>pH_{24}</th>
<th>CFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>6.83</td>
<td>6.81</td>
<td>&gt;10^7</td>
</tr>
<tr>
<td>DHAA (5 mM)</td>
<td>6.58</td>
<td>6.18</td>
<td>&gt;10^7</td>
</tr>
<tr>
<td>Nisin (100 IU/ml)</td>
<td>6.76</td>
<td>6.72</td>
<td>&gt;10^7</td>
</tr>
<tr>
<td>DHAA + nisin</td>
<td>6.57</td>
<td>6.15</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>
Table 4.4 *Listeria* growth in meat extract after 48 h at 30 °C. The protective effect of AA, sodium nitrite and their combination on nisin stored for 24 h at 20 °C prior to inoculation.

<table>
<thead>
<tr>
<th>Test Combinations</th>
<th>pH&lt;sub&gt;initial&lt;/sub&gt;</th>
<th>pH&lt;sub&gt;24&lt;/sub&gt;</th>
<th>CFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>6.62</td>
<td>6.67</td>
<td>&gt;10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>In situ</em> DHAA (10 mM)</td>
<td>6.35</td>
<td>5.91</td>
<td>&gt;10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nisin (100 IU/ml)</td>
<td>6.61</td>
<td>6.66</td>
<td>&gt;10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>In situ</em> DHAA + nisin</td>
<td>6.37</td>
<td>6.03</td>
<td>&lt;10</td>
</tr>
<tr>
<td>AA + nisin</td>
<td>6.15</td>
<td>6.22</td>
<td>&lt;10</td>
</tr>
<tr>
<td>SN + nisin</td>
<td>6.57</td>
<td>6.59</td>
<td>&gt;10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>AA (10 mM)</td>
<td>6.19</td>
<td>6.26</td>
<td>&gt;10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>SN (20 mM)</td>
<td>6.59</td>
<td>6.62</td>
<td>&gt;10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
4.3.9 Bactericidal effect of nisin and/or DHAA in meat slurry

The bactericidal effect of nisin and its combination with DHAA on the total viable and *L. monocytogenes* populations in raw meat slurry was investigated. The slurry was prepared by mixing minced beef meat with an equal quantity of water (1:1) and nisin/DHAA. After 12 hours storage at 4°C the *Listeria* suspensions were added at levels of 6.5 log CFU/g. Total viable counts and pH values were estimated immediately after the mixing of the slurry and at the end of the 12-hour incubation at 4°C.

As shown in Table 4.5 the pH of all samples ranged between 5.27 and 5.57 and remained constant over the next 12 hours. Initial total viable microbial population was estimated at about 6.5 log CFU/g for all tests. In the control slurry it increased by approximately 0.5 log CFU/ml after 12 hours of storage at 4°C. At the same time, total viable counts for nisin alone and its combination with DHAA were 6.05 and 5.85 log CFU/g respectively, a decrease that was probably due to the reduction in the numbers of Gram-positive bacteria. Initial estimation of lactic acid bacteria on MRS agar was approximately $10^6$ CFU/g and decreased by approximately 1 log CFU/g after 12 hours (results not shown). Dehydroascorbic acid on its own had no effect on the total viable counts.

Table 4.5 Total viable counts and pH values of meat slurry samples estimated immediately after the addition of the compounds and after 12 hours of incubation at 4°C.

<table>
<thead>
<tr>
<th>Test Combinations</th>
<th>0 h TVC (log CFU/g)</th>
<th>pH ± 0.20</th>
<th>12 h TVC (log CFU/g)</th>
<th>pH ± 0.30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisin + DHAA</td>
<td>6.62</td>
<td>5.32 ± 0.20</td>
<td>5.85</td>
<td>5.27 ± 0.30</td>
</tr>
<tr>
<td>Nisin (100 IU/g)</td>
<td>6.57</td>
<td>5.57 ± 0.10</td>
<td>6.05</td>
<td>5.58 ± 0.10</td>
</tr>
<tr>
<td>DHAA (5 mM)</td>
<td>6.71</td>
<td>5.27 ± 0.30</td>
<td>6.54</td>
<td>5.29 ± 0.20</td>
</tr>
<tr>
<td>No addition</td>
<td>6.65</td>
<td>5.57 ± 0.10</td>
<td>7.14</td>
<td>5.60 ± 0.05</td>
</tr>
</tbody>
</table>
As shown in Figure 4.19 storage of the nisin-containing meat slurry at 4°C for 12 hours prior to inoculation with \textit{L. monocytogenes}, reduced the bactericidal effect of nisin compared to nisin added just before inoculation. After two hours, surviving populations of \textit{L. monocytogenes} were present at levels of 4.7 log CFU/g and 2.1 log CFU/g respectively. This indicates that over the last 12 h pre-incubation nisin had partly reacted with the free \(-\text{SH}\) groups or was consumed for the inactivation of the Gram-positive bacteria present in the meat.

Introduction of a commercial DHAA preparation into the meat system prior to the addition of the nisin was shown to restore nisin activity. Exposure of \textit{L. monocytogenes} to this combination for two hours produced similar count reduction to that when nisin was added before inoculation with the test strain.

Dehydroascorbic acid added on its own had no effect on the survival of \textit{L. monocytogenes}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure4.19.png}
\caption{Effect of nisin (100 IU/ml) on \textit{L. monocytogenes} NCTC in meat slurry stored for 12 hours at 4°C in the presence of commercial DHAA. Each point represents the mean ± standard deviation of two independent experiments tested in duplicate.}
\end{figure}
4.3.10 Bactericidal effect of nisin and/or in situ produced DHAA in meat slurry

The bactericidal effect of nisin and its combination with in situ produced dehydroascorbic acid was also investigated in raw meat slurry after a 12 hour-storage at 4°C prior to inoculation. Total viable counts and pH values were estimated immediately after the mixing of the slurry and at the end of the 12-hour incubation at 4°C.

As shown in Table 4.6 initial pH ranged between 5.09 (for nisin plus ascorbic acid) and 5.57 (no addition) and no changes were observed thereafter. Initial microflora levels were approximately 6.6 log CFU/g. After 12 hours of storage, microbial counts differed with the combinations used. Ascorbic acid, sodium nitrite alone and in combination had no effect on microbial populations. Nisin alone produced about 0.5 log cfu/g reduction, but more than 1 log cycle reduction in the presence of AA. The combination of nisin with sodium nitrite and in situ produced DHAA caused similar reduction to nisin alone.

Table 4.6 Total viable counts and pH values of test samples estimated immediately after the addition of the compounds and after 12 hours of incubation at 4°C.

<table>
<thead>
<tr>
<th>Test Combinations</th>
<th>0 h</th>
<th>12 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisin + In situ DHAA</td>
<td>6.65 ± 0.03</td>
<td>5.85 ± 0.10</td>
</tr>
<tr>
<td>Nisin + AA</td>
<td>6.66 ± 0.10</td>
<td>5.55 ± 0.30</td>
</tr>
<tr>
<td>Nisin + SN</td>
<td>6.64 ± 0.10</td>
<td>6.29 ± 0.10</td>
</tr>
<tr>
<td>Nisin (100 IU/g)</td>
<td>6.57 ± 0.20</td>
<td>6.05 ± 0.20</td>
</tr>
<tr>
<td>AA (10 mM)</td>
<td>6.63 ± 0.20</td>
<td>6.51 ± 0.10</td>
</tr>
<tr>
<td>SN (20 mM)</td>
<td>6.64 ± 0.20</td>
<td>6.67 ± 0.05</td>
</tr>
<tr>
<td>In situ DHAA (10 mM)</td>
<td>6.66 ± 0.05</td>
<td>6.66 ± 0.20</td>
</tr>
<tr>
<td>No addition</td>
<td>6.65 ± 0.05</td>
<td>7.14 ± 0.05</td>
</tr>
</tbody>
</table>
When *in situ* produced dehydroascorbic acid was present with nisin in the meat slurry during pre-incubation, it improved nisin activity against added *L. monocytogenes*. Enumeration of surviving populations two hours after inoculation showed that the presence of the DHAA reduced counts by 2.96 log cycles, approximately 0.5 log cycles more than nisin alone.

The presence of ascorbic acid significantly enhanced nisin activity and the count reduction produced after 2 hours was very similar to that caused by nisin added just before inoculation. Mean log reduction for these two samples was 4.69 log CFU/ml. Ascorbic acid on its own had no effect on survival of the test strain.

The addition of sodium nitrite in the nisin-containing meat slurry produced no additional bactericidal effect and when added on its own had no effect at all on *Listeria* counts (Fig 4.20).

![Graph showing the effect of nisin (100 IU/ml) on *L. monocytogenes* in meat slurry stored for 12 hours at 4°C in the presence of AA (10 mM), SN (20 mM) and their combination (*in situ* produced DHAA). Each point represents the mean ± standard deviation of two independent experiments tested in duplicate.]

**Figure 4.20** Effect of nisin (100 IU/ml) on *L. monocytogenes* in meat slurry stored for 12 hours at 4°C in the presence of AA (10 mM), SN (20 mM) and their combination (*in situ* produced DHAA). Each point represents the mean ± standard deviation of two independent experiments tested in duplicate.
4.3.11 Bactericidal effect of nisin and/or commercial DHAA in cooked meat slurry

In order to evaluate the protective effect of DHAA in a situation closer to commercial practice the growth of a cocktail of *Listeria monocytogenes* strains in a pasteurised meat slurry over a period of 33 days at 8°C was monitored. All additions of nisin and dehydroascorbic acid were made simultaneously 30 minutes before thermal processing. Results (Table 4.7, Fig. 4.21) showed that the addition of nisin alone (100 IU/ml) reduced populations by two log cycles, but survivors recovered to initial inoculum levels within 11 days, with populations estimated at $10^6$ CFU/g after 17 days of storage. DHAA added on its own (2.5 mM) slightly retarded growth by two days compared to the control. Nevertheless, its combination with nisin totally suppressed growth and after 33 days of storage with counts remaining below $10^2$ CFU/g. pH was similar for all tests (5.6 to 5.8), showing that improved nisin activity was not the result of the low pH which is known to enhance its activity.

Table 4.7 Rate of *Listeria* growth in pasteurised meat slurry at 8 °C. Enhancement of nisin activity by DHAA.

<table>
<thead>
<tr>
<th>Additions to meat</th>
<th>pH</th>
<th>Days until <em>Listeria</em> counts reached $10^6$ CFU/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no addition)</td>
<td>5.80</td>
<td>5</td>
</tr>
<tr>
<td>DHAA at 2.5 mM</td>
<td>5.63</td>
<td>7</td>
</tr>
<tr>
<td>Nisin at 100 IU/g</td>
<td>5.76</td>
<td>17</td>
</tr>
<tr>
<td>DHAA + nisin</td>
<td>5.61</td>
<td>&gt; 33</td>
</tr>
</tbody>
</table>
Figure 4.21 Growth of *L. monocytogenes* (strains 272, 358, NCTC12426 and CRA3930) in cooked meat slurry stored at 8°C in the presence of nisin (100 IU/ml) and or DHAA (2.5 mM). Additions were made before heat processing at 90°C for 10 min. (Minimum detection limit 100 CFU/g apart from days 0 and 1 at 10 CFU/g). Arrows mark undetectable counts.
4.3.12 Bactericidal effect of nisin and/or *in situ* produced DHAA in meat slurry

A similar experiment to that described in the previous section was performed where ascorbic acid, sodium nitrite alone or in combination, were added to the pasteurised meat slurry system with nisin.

Results are summarised in Table 4.8 and shown in Figure 4.22. Ascorbic acid alone did not inhibit *Listeria*. Nisin and sodium nitrite delayed *Listeria* growth reaching the threshold of $10^6$ CFU/ml after 6 days and 10 days respectively. The double combinations (ascorbic acid plus sodium nitrite, sodium nitrite plus nisin, ascorbic acid plus nisin) were all more effective, particularly that of ascorbic acid plus sodium nitrite. However, the triple combination (ascorbic acid plus sodium nitrite plus nisin) achieved the best inhibition, which was taken as evidence of the nisin-protective benefit of DHAA produced *in situ* by nitrite oxidation of AA.

**Table 4.8** Rate of *Listeria* growth in pasteurised meat slurry at 8 °C. Enhancement of nisin activity by AA, SN and their combination.

<table>
<thead>
<tr>
<th>Additions</th>
<th>pH</th>
<th>Days until <em>Listeria</em> counts reached $10^6$ CFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>6.33</td>
<td>4</td>
</tr>
<tr>
<td>Ascorbic acid at 5 mM</td>
<td>5.96</td>
<td>4</td>
</tr>
<tr>
<td>Sodium nitrite at 10 mM</td>
<td>6.33</td>
<td>10</td>
</tr>
<tr>
<td>Nisin at 100 IU/g</td>
<td>6.20</td>
<td>6</td>
</tr>
<tr>
<td>AA + SN</td>
<td>6.09</td>
<td>16</td>
</tr>
<tr>
<td>Nisin + AA</td>
<td>6.01</td>
<td>10</td>
</tr>
<tr>
<td>Nisin + SN</td>
<td>6.20</td>
<td>12</td>
</tr>
<tr>
<td>Nisin + AA + SN</td>
<td>6.10</td>
<td>26</td>
</tr>
</tbody>
</table>
Figure 4.22 Growth of *L. monocytogenes* (strains 272, 358, NCTC12426 and CRA3930) in cooked meat slurry stored at 8°C in the presence of nisin (100 IU/ml) and or AA (5 mM) and NaNO₂ (10 mM). Additions were made before heat processing at 90°C for 10 min. (Minimum detection limit 100 CFU/g apart from days 0 and 1 at 10 CFU/g). Arrow marks undetectable counts.
4.3.13 Conversion of GSH to GSSG in the presence of AA and DHAA

The fate of free glutathione in the presence of DHAA and AA in 50 mM phosphate buffer pH 6.5 and BHI broth at 30°C was assessed using Ellman's method (1959). As shown in Figures 4.23a and 4.23b in both systems examined, GSH alone decreased at rates similar to those seen in the presence of DHAA. Ascorbic acid slightly delayed the reduction in the free thiol content, probably because it was acting as an antioxidant protecting the glutathione.

The rate of loss of free sulfhydryl group was much higher in BHI broth, where levels decreased by about 70% in 100 minutes compared to 5-6 hours in phosphate buffer. This may be due acceleration of autoxidation by components of the BHI broth or reaction of glutathione with BHI components (Figures 4.23a,b).

**Figure 4.23** Reduction in the free thiol content (free glutathione, 2 mM) in the presence of DHAA (1 mM) and ascorbic acid (1 mM) in a) BHI broth and b) phosphate buffer (pH 6.5, 50 mM).
4.4 DISCUSSION

The results of this study clearly indicate that in model system experiments, the minimum inhibitory concentration of nisin required to inhibit the growth of *L. monocytogenes* was significantly increased by the addition of glutathione due to the formation of the nisin-glutathione adduct. This has confirmed the previous observations of Rose *et al.*, (1999; 2000). The proposed sites of glutathione addition on the nisin molecule are the dehydrated amino acids (Rose *et al.*, 2003), which are known to be essential for complete retention of nisin activity (Chan *et al.*, 1996; Rollema *et al.*, 1996). Therefore, the interaction of nisin with glutathione renders the nisin molecule inactive against its target microflora. The introduction of dehydroascorbic acid into the nisin-glutathione system prior to the addition of nisin was shown to restore nisin activity possibly through a protective effect, as shown by the subsequent reduction of the minimum inhibitory concentration to levels similar to those seen with nisin alone. Dehydroascorbic acid on its own did not have an inhibitory effect at the levels used.

Removing glutathione from model systems using dehydroascorbic acid was further examined with regard to its stimulatory effect on the bactericidal activity of nisin. In the presence of dehydroascorbic acid the bactericidal effect of nisin was restored or even enhanced depending on the concentration of dehydroascorbic acid. However, dehydroascorbic acid is not permitted as a food additive by European legislation and its use as a commercial preparation is also uneconomic.

Ascorbic acid is often added during curing of meats and its oxidation to dehydroascorbic acid by sodium nitrite and other heme-containing compounds contributes to colour improvement and stabilisation. In this study we produced dehydroascorbic acid via oxidation of ascorbic acid by sodium nitrite and its effect on nisin activity in the presence of GSH was assessed. Our results showed that the *in situ* produced DHAA also restored nisin activity in the presence of GSH but its protective effect was less compared to the commercial preparation. This may be due to lower concentrations of DHAA produced by *in situ* oxidation.

However, the combination of nisin with ascorbic acid, without nitrite, in the presence of glutathione also produced a synergistic effect against *L. monocytogenes*, even though it
would be expected that nisin would be inactivated due to the formation of the nisin-glutathione adduct. It may be that ascorbic acid is oxidised to dehydroascorbic under aerobic conditions. The fact that the count reduction in the presence of ascorbic acid was similar to that obtained with commercial and in situ produced dehydroascorbic acid might support the view that the ascorbic acid has been oxidised to dehydroascorbic acid.

There are however, problems with that explanation. The conversion of glutathione to its dimeric form by dehydroascorbic acid has been previously reported. In dough mixing inactivation of free glutathione is assisted by the addition of ascorbic acid which is oxidised to dehydroascorbic acid and this, in the presence of the enzyme glutathione hydrogenase, dimerises glutathione (Every 1996; Coultrate, 2000). In other studies it has been reported that the spontaneous, non-enzymatic reaction between the two components does occur (Winkler 1987; Park, 2001). Indeed, assuming that dehydroascorbic acid converts reduced glutathione to its disulfide, a subsequent reduction in the free thiol content would be expected. However, our results showed that the rate of reduction of free glutathione in the presence of ascorbic acid and dehydroascorbic acid was similar to that seen with glutathione alone. Therefore, it seems that the decrease observed was not due to oxidation of glutathione by dehydroascorbic acid, but probably due to an autoxidation process in the presence of oxygen. Indeed, its facile electron-donating capacity makes glutathione a very important water-phase antioxidant providing protection for cells against endogenous oxygen radicals. The rate of glutathione autoxidation was shown to be higher in BHI broth, suggesting that other components, eg. metal ions, may be involved in the inactivation process, either by accelerating autoxidation or by introducing additional binding sites for the tripeptide.

Also, if dehydroascorbic acid is able to oxidise glutathione, sodium nitrite being a strong oxidising agent would be expected to have the same effect. The free-energy change of the above oxidation-reduction reactions can be readily calculated from the difference in reduction potentials of the reactants (Stryer, 2000):

\[
\text{DHAA} + 2\text{GSH} \rightarrow \text{AA} + \text{GSSG}
\]

The reduction potential of DHAA : AA couple is 80 mV (a)
The reduction potential of GSH (oxidised): GSH (reduced) couple is -230 mV (b)
Therefore, writing the above reactions as reductions: oxidant $\rightarrow e^- +$ reductant

Equation (a) becomes \( \text{DHAA} + 2\text{H}^+ + 2e^- \rightarrow \text{AA} \) \((E_0' = 80 \text{ mV})\)

Similarly,

Equation (b) becomes \( 2\text{GSH} \) (oxidised) $+ 2\text{H}^+ + 2e^- \rightarrow \text{GSH} \) (reduced) \((E_0' = -230 \text{ mV})\)

Subtracting reaction (b) from reaction (a) yields the desired reaction, and \( \Delta E_0' = 350 \text{ mV} \)

The standard free energy change \( \Delta G^o = -nF\Delta E_0' \)
Where, \( n \) is the number of electron transferred and \( F = 23.06 \text{ kcalV}^{-1}\text{mol}^{-1} \)
And so \( \Delta G^o = -2 \times 23.06 \times 0.35 = -16 \text{ kcal/mol} \).

A negative \( \Delta G^o \) signifies an exergonic reaction, hence a reaction that can occur spontaneously (Stryer, 2000).

In a similar way, calculation of the free energy change for the reaction between nitrite and glutathione gives a value of \( \Delta G^o = -31 \text{ kcal/mol} \), also indicating that this reaction happens spontaneously. However, the slight logarithmic reduction (less than 1 log CFU/ml) seen in the presence of nisin, glutathione and sodium nitrite suggest that glutathione was not inactivated in the presence of sodium nitrite. This is further indication that the inactivation of glutathione is not due to the presence of oxidising compounds, such as dehydroascorbic acid and nitrite.

Although ascorbic and dehydroascorbic acid were not shown to directly remove glutathione as hypothesised initially, their presence did inhibit the effect of glutathione either by preventing formation of the nisin-GSH adduct or by enhancing the activity of residual nisin. This remains an area for future investigation.

Similar to model systems, the protective effect of ascorbic and dehydroascorbic acid on nisin activity was also seen when this work was transferred to raw meat extract as well as to raw and heat-processed meat slurry. Our results showed that nisin activity in meat decreased during storage, due to interactions with meat compounds (e.g., glutathione or...
other free thiol compounds), but the addition of reduced and oxidised ascorbic acid was able to prevent their inhibitory effect.

The use of natural preservatives like nisin as partial replacement of nitrates is desirable, but its variable effectiveness demonstrated in numerous studies makes its application in meat products rather problematic. Even though glutathione has been proposed as a binding site for nisin in fresh meat (Rose et al., 1999) it can not entirely account for its poor antimicrobial performance in meat products, since a number of other possible reasons for this have been identified (Aasen et al., 2003; Bell and deLacy, 1986; Coventry at al., 1995; Davies et al., 1999; Jaber et al., 2000). However, the combination of nisin with ascorbic or dehydroascorbic acid may make this system a potential candidate for the inhibition of food-borne pathogens in meat products, particularly at chill temperatures.
CHAPTER 5

SYNERGISTIC EFFECT OF NISIN AND ANTIOXIDANTS ON
L. MONOCYTOGENES
CHAPTER 5

SYNERGISTIC EFFECT OF NISIN AND ANTIOXIDANTS ON L. MONOCYTOGENES

5.1 INTRODUCTION

Fats and oils are one of the main constituents of foods and one of the basic nutrients in human diet. Oxidation is generally regarded as the most important mode of lipid deterioration leading to rancidity and product rejection. It takes place in a stepwise fashion as an autocatalytic reaction proceeding at an increasing rate, involving three stages: 1. Initiation - formation of free radicals, 2. Propagation - free-radical chain reaction, 3. Termination - formation of non-radical products (Coultate, 1996; Gordon, 2001)

Lipid oxidation can be controlled or minimised by the use of synthetic or natural food antioxidants (Davidson, 1983; Raccach, 1984). Phenolics are antioxidants with redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Proestos et al., in press). Phenolic antioxidants are able to inhibit or interfere with the free-radical autoxidation reaction, fundamental to lipid oxidation. They have the ability to suppress the initial fatty free radical (R'), thus delaying the onset of the autoxidation process in fat or oil. However, concern about the safety of synthetic antioxidants has led to increasing use of substances from vegetable sources as antioxidants in foods.

Of the many natural antioxidants, tocopherols are widely distributed and most extensively tested for antioxidant activity but their antioxidant activity is not as effective as the synthetic phenolic compounds (Cuppert, 2001; Shi et al., 2001). Rosemary extract has been shown to have significant antioxidant activity. Rosemariquinone and rosemari diphenol are the two antioxidant constituents with potency equal or superior to that of BHA and BHT.
Spoilage and pathogenic microorganisms, including *L. monocytogenes*, *Salmonella*, *Staph. aureus*, *Pseudomonas*, *Bacillus*, *E. coli* and clostridia have been shown to be inhibited by synthetic and natural phenolic antioxidants used individually and in combination with other antimicrobial agents in foods and food model systems (Davidson *et al.*, 1981; Eubanks and Beuchat, 1982; Shelef and Liang 1982; Payne *et al.*, 1989; Yousef *et al.*, 1991; Oluyemi *et al.*, 1996; Pol and Smid, 1999; Ultree, 2000). In general, Gram-positive bacteria have been found to be more affected than Gram-negative, but differences have also been noted among strains of the same species (Fung *et al.*, 1985). Variation in the antimicrobial properties of phenolic antioxidants has been attributed to numerous factors, including the type and the concentration of the compounds, the concentration of the microbial challenge, storage conditions and food composition (Raccach, 1984; Pandit and Shelef, 1994; Skandamis and Nychas, 2000).

Recent studies have shown that the combination of nisin with other antimicrobial agents can extend nisin activity against Gram-negative bacteria (Stevens *et al.*, 1991; Cutter and Siragusa, 1995a; Scannell *et al.*, 1997; Boziaris and Adams, 1999; Ariyapitiptun *et al.*, 1999) and enhance nisin protection against relatively resistant Gram-positive bacteria. *Listeria monocytogenes* has been reported to exhibit considerable resistance/tolerance towards nisin (Davies and Adams, 1994), but its combined use with sucrose fatty acid esters (Thomas *et al.*, 1998), thymol (Ettayebi *et al.*, 2000) and garlic extract (Singh *et al.*, 2001) have been shown to be possible ways to overcome this problem.

The aim of this study was to quantify the individual and combined inhibitory effect of nisin and different commercial antioxidants on *L. monocytogenes* NCTC5105 in laboratory media and UHT milk, and to investigate further the possible mechanism of synergy between these components.
5.2 MATERIALS AND METHODS

5.2.1 Cultures

For all experiments in this study, the test microorganisms used were *Listeria monocytogenes* NCTC5105 and *Micrococcus luteus* NCIB 8166. Resuscitation was performed as described in Chapter 2, General Materials and Methods. All strains were grown at 30°C in BHI broth for 15-18 hours to reach stationary phase, prior to each experiment.

5.2.2 Nisin

See Chapter 2, General Materials and Methods

5.2.3 Antioxidants

All antioxidants used for this study were kindly provided by Danisco Denmark. Stock solutions of 100,000 ppm were generated by dissolving 1 g of each antioxidant in 10 ml of absolute ethanol.

The composition of the antioxidants used was as follows:

1. **Grindox Propyl Gallate**: composed of Propyl gallate (E310) 98%
2. **Guardian Rosemary Extract**: composed of
   - Phenolic diterpenes (compound present in rosemary extract) 4%
   - Maltodextrin and Natural rosemary extract 96%
3. **Grindox 121**: composed of
   - Butylated hydroxyanisole (BHA)(E320) 20%
   - Butylated hydroxytoluene (BHT)(E321) 20%
   - Mono- and diglycerides of fatty acids (E471) and Diacetyl tartaric acid esters of mono- and diglycerides of fatty acids (E472) 60%
4. **Grindox 1029**: composed of
   - Ascorbyl palmitate (E304) 20%
5. Grindox Toco 70: composed of
- Natural mixed tocopherols (E306) 70%
- Vegetable oil 30%

6. Grindox BHT: composed of Butylated hydroxytoluene (BHT) (E321) 99%

All percentages are by weight.

5.2.4 Minimum Inhibitory Concentrations for antioxidants

In BHI broth, appropriate volumes of each antioxidant stock solutions were added in order to achieve final concentrations, which were as follows:

- Grindox Propyl Gallate: 20, 50, 100, 150, 200 ppm
- Guardian Rosemary Extract: 200, 350, 500, 750 ppm
- Grindox 121: 100, 250, 500, 750, 1000 ppm
- Grindox 1029: 200, 350, 500, 750, 1000 ppm
- Grindox Toco 70: 100, 250, 500 ppm
- Grindox BHT: 20, 50, 100, 150, 200 ppm

An overnight culture of the bacterial strain was serially diluted in MRD and aliquots of 0.1 ml of were used to inoculate the broths to generate an initial population of approximately 10^3 CFU/ml. All tests had a final volume of 10 ml and were performed in triplicate. Antioxidant-free ethanol controls were also set up. Test tubes were incubated at 30°C and observed for turbidity after 24 and 48 hours. The MIC was determined by the antioxidant concentration at which bacterial growth was fully inhibited. The addition of Grindox 1029, Grindox 121 and Toco 70 led to instant turbidity; hence, determination of the MIC was achieved using the Miles-Misra technique. Briefly, 1 ml of each test tube for all antioxidant levels was taken and serially diluted in MRD. A volume of 20 μl of each dilution was placed onto BHI agar. The plates were then incubated at 30°C for 24 hours. The MIC limit was set at 10^5 CFU/ml. Counts at this level have been found to correlate with visual determination of growth (Appendix 1). Sampling times were similar to those used for visual MIC determination. Initial inoculum levels were obtained by count enumeration on BHI agar.
5.2.5 Minimum Inhibitory concentrations for the combination of nisin and antioxidants

MICs were performed in BHI broth. A range of nisin concentrations (0-1000 IU nisin/ml) were set up by adding 1ml of the prepared nisin stock solutions to the broths containing antioxidants (highest recommended level). An overnight culture of the test strain was serially diluted in MRD and aliquots of 0.1ml of were used to inoculate the broths to generate an initial population of approximately $10^3$ cfu/ml. All tests had a final volume of 10ml and were performed in triplicate. Control samples containing only nisin and antioxidant-free methanol controls were also set up. Tests were incubated at 30°C and observed after 24 and 48 hours. The MIC was determined by the nisin concentration at which bacterial growth was fully inhibited. For Grindox 1029, Grindox 121 and Toco 70 the determination of the minimum inhibitory concentration was achieved by viable count enumeration using the Miles-Misra technique.

5.2.6 Bactericidal effect of combinations of nisin with antioxidants

In this experiment, the bactericidal effect of a two-way combination of nisin with three different antioxidants was evaluated. In BHI broth (50 ml) appropriate volumes of the nisin and antioxidant stock solutions were added to generate final experimental conditions of 200 IU nisin/ml, 200 ppm propyl gallate, 200 ppm BHT and 750 ppm rosemary extract. Controls consisting of sole additions of nisin and antioxidants were also set up.

An appropriate volume of an overnight culture of *Listeria* was added to give an initial inoculum of approximately $10^8$ CFU/ml. The pH of all test samples had been previously recorded and was found to be 7.2 ± 0.1.

Sampling was performed at the following intervals; 0, 5, 15, 30 60, 90 and 120 minutes. At these times a 1ml aliquot of each test was used to set up a dilution series in MRD. Then, 20 μl drops of each dilution were placed in quadruplicate onto BHI agar. All plates were incubated at 30°C for 24 hours, and then counted for colonies. These counts were then converted into CFU/ml for each stage of the experiment in order to be plotted as survivor curves.
5.2.7 The inhibitory effect of combinations of nisin with antioxidants

In the first series of these experiments, the bactericidal effect of a two-way combination of nisin with seven different antioxidants was evaluated. In BHI broth (50 ml) appropriate volumes of the nisin and antioxidant stock solutions were added to generate final experimental conditions of 200 IU nisin/ml, 200 ppm propyl gallate, 200 ppm BHT and 750 ppm rosemary extract, 1 and 10 mM of ascorbic acid, 500 ppm Toco 70 and 1000 ppm Grindox 121. Controls consisted of single additions of nisin and antioxidants were also set up.

An appropriate volume of an overnight culture of *Listeria* was added to give an initial inoculum of approximately $10^4$ CFU/ml. The pH of all test samples had been previously recorded and was found to be $7.2 \pm 0.1$.

Sampling was performed at regular intervals. At these times a 1ml of each test was used to set up a dilution series in MRD and 100 μl aliquots of the last two dilutions were placed in duplicate onto BHI agar. All plates were incubated at 30°C for 24 hours and 8°C for 28 days, and then counted for colonies of *L. monocytogenes*.

For the experiments investigating the effect of sequential treatments with antioxidants and nisin on the growth of *L. monocytogenes* the following protocols were employed; Either the test strain was initially exposed to nisin and after one hour suspensions were transferred to fresh BHI broth containing different antioxidants, or suspensions were initially exposed to the antioxidants for three hours prior to their transfer to fresh BHI broth where nisin had been added. The antioxidants used were propyl gallate, BHT rosemary extract and ascorbic acid. Samples where cells were not treated prior to or after their transfer to the fresh medium were also prepared and served as controls. Transfer to fresh medium was achieved by diluting properly the treated suspensions to achieve populations of approximately $10^4$ in the fresh medium. Sampling and estimation of counts was carried out similarly to the first experiment.
5.2.8 Evaluation of antioxidant activity

The effect of each antioxidant on the 2, 2-diphenyl-1-picrylhydrazyl (DPPH') radical was estimated according to the procedure described by Brand-Williams et al., (1995). An aliquot of methanol (0.1 ml) solution containing different antioxidants at three different levels, including those where no inhibition of microbial growth was observed, was added to 3.9 ml of DPPH' 0.025g litre\(^{-1}\) in methanol prepared daily. Absorbance at 515 nm for each sample was measured every minute for the first 5 minutes and in short time intervals until the reaction reached a plateau. The DPPH' concentration was calculated from a calibration curve with the equation:

\[
[DPPH'] = (Abs_{515} + 0.00258) \times \text{Dilution factor / 12,509}
\]

The percentage of remaining DPPH' (%DPPH\(_{\text{REM}}\)) was calculated for each antioxidant tested according to the following equation and the reaction kinetics were plotted.

\[
\%\text{DPPH}_{\text{REM}} = \left(\frac{[DPPH']_T}{[DPPH']_T=0}\right) \times 100
\]

The time needed for each antioxidant to reduce the DPPH' radical to 50% was estimated graphically.

The percentage of remaining DPPH concentration was plotted against antioxidant concentration for each antioxidant separately and the slope of the standard curve was calculated. Antiradical activity EC\(_{50}\) was defined as the amount of the antioxidant needed to decrease the initial DPPH concentration by 50%. The time T\(_{EC50}\) needed for this reduction was also calculated and the antiradical efficiency was calculated using the equation (Sanchez-Moreno et al., 1997):

\[
AE = \frac{1}{EC_{50} \times T_{EC50}}
\]
5.2.9 Assessment of redox potential in laboratory media containing antioxidants

Redox potential and pH were measured at 30°C. Redox potential was measured using a platinum combination electrode and redox meter (model HI 8424, HANNA instruments, Leighton Buzzard, UK). The response of the electrode was checked regularly in quinhydrone-saturated buffers at pH 4 and 7. pH was measured using a combination pH electrode (PHP-100-030C) and pH meter (Griffin model 80, PHJ-300-010G, Griffin, UK). Redox potentials (E<sub>b</sub>) corrected to pH 7, were calculated using the equation:

\[ E_b = E_{obs} + E_{ref} + EN(pH - 7) \]

Where \( E_{obs} \) is the observed redox potential, \( E_{ref} \) is the redox potential of the internal electrolyte (3.5 M KCl silver/silver chloride) of the electrode taken as 195.6 mV, \( EN \) is the Nernst potential, taken as 61.51 mV (Midgley and Torrance, 1978).

5.2.10 Effect of simultaneous exposure to nisin and propyl gallate in UHT milk

Whole and skimmed UHT milk purchased from a local supermarket were transferred aseptically to sterile Duran bottles and nisin and propyl gallate stock solutions were added to give final concentrations of 200 IU/ml and 200 ppm respectively. Test samples containing only one or none of the two compounds were also prepared and used as controls in order to assess the effect of their combination on Listeria monocytogenes NCTC5105, which had been grown overnight at 30°C in BHI broth and diluted properly to give an initial count of approximately 4 log cfu ml<sup>-1</sup>. Samples were incubated statically under aerobic conditions at 8°C. Growth was monitored by viable count enumeration using the spread plating technique.
5.2.11 Minimum Inhibitory Concentrations of nisin against *L. monocytogenes* in the presence of vegetable oil

MICs were performed in BHI broth. A range of nisin concentrations (0-1750 IU nisin/ml) were set up by adding 1ml of the prepared nisin stock solutions to the broths containing 2.5 and 5 ml of vegetable oil. Mixing of the compounds was obtained by vortexing at high speed for 60 sec. An overnight culture of the test strain was serially diluted in MRD and aliquots of 0.1ml of were used to inoculate the broths to generate an initial population of approximately $10^3$ cfu/ml. All tests had a final volume of 10 ml and were performed in triplicate. Control samples containing nisin only were also set up and were incubated both under aerobic and anaerobic conditions. All tests and controls were incubated at 30°C and observed for turbidity after 24 and 48 hours. The MIC was determined by the nisin concentration at which bacterial growth was fully inhibited.

5.2.12 Nisin activity in an oil-nisin solution emulsion

A nisin solution of 100 IU/ml was prepared by diluting properly the nisin stock solution. Representative 10 ml of that solution were kept in order to determine nisin levels before emulsification. In a separation funnel of 100 ml appropriate volumes of the nisin solution and the vegetable oil were added to generate two mixtures of 1:1 and 3:1 volumes nisin:oil. The mixture was then shaken vigorously for one and a half minutes to achieve emulsification. The emulsion was left to settle down until the aqueous phase was separated completely from the lipid phase. The aqueous phase was collected in a 50 ml glass beaker and a representative sample of 10 ml was taken and assayed for the determination of the nisin content after emulsification.

Samples collected before and after emulsification, were diluted with 90 ml deionised water and their pH was adjusted at 4 ± 0.05. Nisin activity was determined using the bioassay method described in Chapter 2 (General Materials and Methods).
5.2.13 Data Analysis

Data on growth curves were fitted using Microfit Software (version 1.0) (Institute of Food Research, UK). This program was also used for the calculation of growth parameters, such as lag phase (t-lag), maximum growth rate (μmax) and generation time (t-g).

For all experiments two independent trials were conducted and measurements were performed in duplicate, unless otherwise stated. Statistical evaluation of results was performed using SPSS package v.11.0 for Windows using ANOVA and Student’s t test.

5.3 RESULTS

5.3.1 Estimation of the antioxidant Minimum Inhibitory Concentrations (MIC) against *L. monocytogenes*

The lowest level of each antioxidant that would totally inhibit growth was determined either visually by turbidity or using the Miles-Misra technique for TOCO 70, Grindox 1029 and Grindox 121. The MIC determination limit for these antioxidants was set at 5 log CFU/ml. This was based on preliminary experiments where visual estimation of growth of *L. monocytogenes NCTC5105* was possible at microbial count levels of approximately 5 log CFU/ml (Appendix 1).

As shown in Figure 5.1, for propyl gallate and BHT after 24 hours of incubation at 30°C turbidity was observed for all the addition levels. Rosemary extract had no inhibitory effect up to 750 ppm but no turbidity was observed at 1000 ppm. For all levels of TOCO 70, and Grindox 1029 counts after 24 hours of incubation at the same temperature exceeded the MIC determination levels, reaching about 9 log CFU/ml. *Listeria* in BHI broth containing different levels of Grindox 121 grew up to a level of approximately 6 log CFU/ml after 24 hours and over 8 log CFU/ml when incubated for another 24 hours. The results showed, that with the exception of rosemary extract, none of the antioxidants used was able to inhibit *Listeria* growth as measured by the MIC determination, even at the highest level of addition.
Figure 5.1 Estimation of the minimum inhibitory concentration for six different antioxidants against *L. monocytogenes* NCTC at 30°C over a period of 24 hours. (Number of replicates=9). Arrows represent counts over (↑) or below (↓) the MIC determination limit.
5.3.2 Estimation of the nisin Minimum Inhibitory Concentrations in the presence of antioxidants against *L. monocytogenes*

The inhibition of *Listeria monocytogenes* NCTC 5105 by the combined action of nisin and antioxidants was investigated (Fig 5.2). For this experiment a range of different nisin concentrations was set up and the highest level of each antioxidant that did not inhibit *Listeria* growth was used. The lowest level of nisin in the presence of antioxidants that was able to totally inhibit growth was determined either visually by turbidity or using the Miles-Misra technique for TOCO 70, Grindox 1029 and Grindox 121. The MIC determination limit for these antioxidants was set at 5 log CFU/ml.

The MIC for nisin alone in most tests was 200 IU/ml (7/9) and 300 IU/ml (6/9) after 24 and 48 hours respectively. When nisin was combined with propyl gallate, BHT and rosemary extract, the MICs were significantly reduced. The MIC for the combination of nisin and propyl gallate was 50 IU/ml in 9 out of 9 tubes after 24 hours and 100 IU/ml after 48 hours in 6 out of 9 tubes. This was also the case with BHT after 48 hours. In the presence of rosemary extract nisin MIC was 50 IU/ml and 150 IU/ml after 24 and 48 hours respectively. Grindox 121 had an MIC of 100 IU/ml after 24 hours in 7 out of 9 tubes but there was no inhibition after 48 hours up to a nisin concentration of 300 IU/ml. Toco 70 and Grindox 1029 showed no inhibition after both 24 and 48 hours, indicating that these antioxidants actually antagonised nisin activity.

5.3.3 Assessment of oxidative stress in laboratory media containing antioxidants

In order to exclude any possibility of oxidative stress caused to *L. monocytogenes* due to the presence of the antioxidants, the redox potential of laboratory medium (BHI) containing seven different antioxidants was measured. Redox potential was calculated for pH 7 at 30°C. No significant changes were observed in the presence of the antioxidants except for ascorbic acid (10 mM) and Grindox 1029 (Table 5.1).
Figure 5.2 Estimation of nisin Minimum Inhibitory Concentration alone and in the presence of the six different antioxidants against *L. monocytogenes* NCTC5105 at 30°C after 24 h (□) and 48 h (■). (Number of replicates=9).

Table 5.1 Redox potential as measured for BHI broth containing various antioxidants at pH 7, 30°C. \(E_h\) values are representative of four measurements.

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Concentration</th>
<th>(E_h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grindox 121</td>
<td>1000 ppm</td>
<td>239.12 ± 6.53</td>
</tr>
<tr>
<td>Toco 70</td>
<td>500 ppm</td>
<td>236.05 ± 1.76</td>
</tr>
<tr>
<td>Propyl gallate</td>
<td>200 ppm</td>
<td>218.65 ± 4.63</td>
</tr>
<tr>
<td>Grindox 1029</td>
<td>1000 ppm</td>
<td>68.87 ± 11.42</td>
</tr>
<tr>
<td>BHT</td>
<td>200 ppm</td>
<td>243.01 ± 2.03</td>
</tr>
<tr>
<td>Rosemary extract</td>
<td>1000 ppm</td>
<td>204.83 ± 7.62</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>10 mM</td>
<td>33.22 ± 3.25</td>
</tr>
<tr>
<td>No addition</td>
<td>-</td>
<td>269.59 ± 8.41</td>
</tr>
</tbody>
</table>
5.3.4 Evaluation of antioxidant activity

Radical scavenging activity for five antioxidants against the stable free radical DPPH· was evaluated spectrophotometrically at 515 nm (Fig. 5.3). Radical scavenging activity is a function of two parameters, the extent to which an antioxidant is able to decrease the initial DPPH· concentration and the time need for this reduction. Classification of antioxidants takes into consideration the calculation of the parameters EC$_{50}$ and T$_{EC50}$, previously described in the Materials and Methods section (5.2.8). As shown in Table 5.2 the most effective antioxidant was propyl gallate exhibiting both highest antiradical efficiency (2.697) and highest reduction of the stable DPPH·. Even though ascorbic acid demonstrated better antiradical efficiency (1.344) compared to tocopherols (0.177), both antioxidants were shown to be moderate radical scavengers with inhibition just over 40%. BHT showing intermediate reaction kinetics reduced the free radical DPPH· to the same levels as tocopherols and AA by the end of incubation. Finally, rosemary extract obtained remarkably lower antiradical activity after 300 min of incubation.

![Antiradical activity kinetics of methanol solutions of seven different antioxidants at ambient temperature.](image)

**Figure 5.3** Antiradical activity kinetics of methanol solutions of seven different antioxidants at ambient temperature. Antiradical activity was determined by measuring the reduction of the stable free radical DPPH absorbance at 515 nm. Each point represents the mean of three independent measurements.
Table 5.2 Kinetic classification of antioxidants. Reaction parameters are representative of 4 independent measurements

<table>
<thead>
<tr>
<th>Concentration (mM or ppm of antioxidant)</th>
<th>Slope</th>
<th>T[DPPH]₀₅₀ (min)</th>
<th>AE (1/EC*TEc)</th>
<th>Remaining DPPH %</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA 1mM</td>
<td>-0.044</td>
<td>2</td>
<td>1.344</td>
<td>44.38</td>
</tr>
<tr>
<td>PG 200 ppm</td>
<td>-0.644</td>
<td>&lt;1</td>
<td>2.697</td>
<td>4.05</td>
</tr>
<tr>
<td>BHT 200 ppm</td>
<td>-0.096</td>
<td>210</td>
<td>0.012</td>
<td>41.92</td>
</tr>
<tr>
<td>RE 750 ppm</td>
<td>nd</td>
<td>&gt;300</td>
<td>nd</td>
<td>82.56</td>
</tr>
<tr>
<td>Toco 70 500 ppm</td>
<td>-0.086</td>
<td>15</td>
<td>0.177</td>
<td>42.22</td>
</tr>
</tbody>
</table>

Nd: Not determined

5.3.5 Bactericidal effect of nisin against L. monocytogenes in the presence of antioxidants

The bactericidal effect of nisin and its combinations with propyl gallate, BHT and rosemary extract was investigated over a period of two hours. As shown in Figure 5.4 in all cases the combination of nisin with the antioxidants did not produce a higher bactericidal effect on L. monocytogenes compared to nisin alone. After the first 5 min of exposure, surviving populations were similar for all combinations. The total reduction in counts after 2 hours was found to be 3.8, 3.7 and 3.4 log CFU/ml for nisin, nisin plus BHT and nisin plus rosemary extract, respectively. None of the antioxidants when used on their own produced any bactericidal effect on Listeria.
Figure 5.4 Bactericidal effect of nisin alone and in the presence of antioxidants against *L. monocytogenes*. Nisin plus antioxidant (*), Nisin (●), antioxidant (▲), No addition (*). Each point represents the mean value ± standard deviation of three independent experiments.
5.3.6 Effect of nisin on the survival and growth of *L. monocytogenes* in the presence of antioxidants

In the first series of experiments inhibition of *L. monocytogenes* NCTC5105 was studied under simultaneous exposure to nisin and seven different antioxidants, in BHI broth at 30°C. Results are depicted in Figure 5.5 (a-g). Growth parameters (No, Nmax, μmax and t-lag) were calculated using Microfit software for treatments where counts were within detection limits.

Exposure of cells to nisin in the presence of propyl gallate was found to have significant effect on cell recovery and growth. After initial cell reduction, population numbers remained below detectable levels (10 CFU/ml) for more than 24 hours of incubation. Counts recovered to initial addition levels after approximately 54 hours, as calculated graphically (Fig. 5.5a). A similar pattern was seen in the presence of BHT, rosemary extract and with 10 mM (but not with 1 mM) of ascorbic acid (Fig. 5.5b, c, d and e). Addition of Toco 70 and Grindox 121 in BHI broth containing nisin, removed totally its antibacterial ability, and in view of this and their complex composition (see section 5.2.3) they were not tested further.

Cell suspensions exposed to nisin alone were able to recover much faster in the absence of antioxidants. Counts recovered to initial levels after approximately 12 hours, and started to grow in rates similar to healthy cells.

Additions of antioxidants alone had a notable effect on growth of *L. monocytogenes*. All growth parameters were affected unfavourably but to a different extent, depending on the antioxidant used (Table 5.3). In all cases lag phase (t-lag) and generation time (t-d) were increased, and maximum growth rate was decreased. Analysis of variance showed that differences were significant (p<0.05). The pattern of effect on growth differed between antioxidants. For example, rosemary extract produced the biggest increase in the lag phase, indicating that at this addition level it can act bacteriostatically. In contrast, Toco 70 had no effect on lag phase but lowered significantly maximum growth rate. This might indicate that the mode of action varies between antioxidants.
Figure 5.5 The effect of the simultaneous exposure to nisin and antioxidants on *L. monocytogenes* NC1C5105 at 30°C. Nisin plus antioxidant (•), Nisin (▲), antioxidant (●), No addition (★). Each point represents the mean± standard deviation of two independent experiments. Arrows mark undetectable counts (<10 CFU/ml).
The effect of the simultaneous exposure to nisin and antioxidants on *L. monocytogenes* NCTC5105 at 30°C. Nisin plus antioxidant (•), Nisin (▲), antioxidant (●), No addition (★). Each point represents the mean± standard deviation of two independent experiments. Arrows mark undetectable counts (<10 CFU/ml).

Figure 5.5 (Continued)
Table 5.3 Growth parameters for *L. monocytogenes* NCTC5105 incubated at 30°C in the presence of different antioxidants. Values represent the mean ± standard deviation of two independent experiments tested in duplicate.

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>N max (log CFU/ml)</th>
<th>t-lag (hours)</th>
<th>μmax (h⁻¹)</th>
<th>t-d (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propyl gallate (1 mM)</td>
<td>6.92 ± 0.31</td>
<td>3.72 ± 0.45</td>
<td>0.43 ± 0.02</td>
<td>1.87 ± 0.11</td>
</tr>
<tr>
<td>BHT (1 mM)</td>
<td>8.13 ± 0.32</td>
<td>3.82 ± 0.12</td>
<td>0.65 ± 0.03</td>
<td>1.39 ± 0.07</td>
</tr>
<tr>
<td>Rosemary extract (750 ppm)</td>
<td>7.20 ± 0.28</td>
<td>13.16 ± 1.68</td>
<td>0.83 ± 0.15</td>
<td>0.86 ± 0.16</td>
</tr>
<tr>
<td>Ascorbic acid (10 mM)</td>
<td>8.34 ± 0.19</td>
<td>2.29 ± 0.12</td>
<td>0.74 ± 0.11</td>
<td>0.82 ± 0.09</td>
</tr>
<tr>
<td>Toco 70 (500 ppm)</td>
<td>8.34 ± 0.19</td>
<td>1.99 ± 0.14</td>
<td>0.41 ± 0.01</td>
<td>1.09 ± 0.03</td>
</tr>
<tr>
<td>Grindox 121 (1000 ppm)</td>
<td>5.08 ± 0.34</td>
<td>7.47 ± 1.26</td>
<td>0.56 ± 0.02</td>
<td>3.75 ± 0.50</td>
</tr>
<tr>
<td>No addition</td>
<td>8.50 ± 0.42</td>
<td>1.89 ± 0.25</td>
<td>0.95 ± 0.02</td>
<td>0.73 ± 0.01</td>
</tr>
</tbody>
</table>
5.3.7 Effect of sequential treatments with antioxidants and nisin on survival and growth of *L. monocytogenes*

Initial treatment with nisin and subsequent transfer of cells into fresh BHI broth containing antioxidants, did not produce the same synergistic effect seen when both antioxidants and nisin were present simultaneously (Figures 5.6a, b, c, d). However, populations injured by nisin were further sensitised by their transfer to media containing BHT (p<0.05) and rosemary extract (p<0.05) compared to nisin treated cells transferred to fresh media containing no antioxidant. This was shown by the calculated t-lag values, Table 5.4.

Slight differences in the recovery and growth profile of injured cells were apparent in the presence of propyl gallate in Figure 5.3, but these were not found to be statistically significant. Treatment with ascorbic acid had no effect on the recovery or growth of injured populations of *L. monocytogenes*, with a mean t-lag = 2.3 hours and maximum growth rate of 0.99 h\(^{-1}\) (Figure 5.6, Table 5.4).

The growth rate of healthy suspensions that were subsequently exposed to the antioxidants was almost identical to that of injured cells exposed to antioxidants. This indicates that the apparent extension of the lag phase and the lower rate of growth were mainly due to the effect of the antioxidants themselves and that nisin treated cells behaved in the same way as untreated cells.

In further experiments investigating the mechanism of synergy, the order of addition was altered and suspensions were exposed to antioxidants prior to treatment with nisin (Fig 5.7a, b, c, d). Over a three-hour period, populations had grown by 0.7 log CFU/ml and 0.3 log CFU/ml in control BHI broth and one supplemented with antioxidants, respectively. Subsequent transfer of untreated cells to nisin solutions produced higher count reduction (4 log cfu/ml) than usually observed. This could be due to the use of the exponential phase cells which are more sensitive to nisin than cells found in stationary phase.

For suspensions treated with antioxidants reduction varied with the antioxidant used. When suspensions were treated with nisin following exposure to rosemary extract, count
reduction was similar to that of healthy cells treated with nisin, but remained below detection levels (<10 CFU/ml) for the next eleven hours. After recovery, they followed a similar growth pattern to nisin treated populations free of antioxidants. With propyl gallate, initial count reduction was similar to that of nisin alone, but there was a short extension of the lag phase (Table 5.5). BHT and ascorbic acid produced a smaller count reduction. The calculated lag phase was also found to be greater (2.09 hours - ascorbic acid, 4.85 hours - BHT) than nisin alone (0.85 hours).
Figure 5.6 Growth of *L. monocytogenes* in BHI broth at 30°C. Nisin (200 IU/ml) treated cells transferred to antioxidants (*•*), Untreated cells transferred to antioxidants (*•*), Nisin treated cells transferred to fresh BHI (*△*), Untreated cells (*•*). Nisin added at -1 hr and transfer to antioxidant at 0 hr. Each point represents the mean± standard deviation of two independent experiments tested in duplicate.
Table 5.4 Growth parameters for *L. monocytogenes* NCTC5105 incubated at 30°C in the presence of different antioxidants following nisin treatment. Values represent the mean ± standard deviation of two independent experiments tested in duplicate.

<table>
<thead>
<tr>
<th>1st Treatment</th>
<th>2nd Treatment</th>
<th>No</th>
<th>Nmax</th>
<th>μmax</th>
<th>t-lag</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(log cfu ml⁻¹)</td>
<td>(log cfu ml⁻¹)</td>
<td>(h⁻¹)</td>
<td>(hours)</td>
</tr>
<tr>
<td>Nisin</td>
<td>Propyl gallate</td>
<td>2.15 ± 0.18</td>
<td>8.74 ± 0.25</td>
<td>0.49 ± 0.04</td>
<td>5.28 ± 1.19</td>
</tr>
<tr>
<td>Nisin</td>
<td>BHT</td>
<td>2.13 ± 0.17</td>
<td>9.59 ± 0.31</td>
<td>0.60 ± 0.40</td>
<td>8.07 ± 0.49</td>
</tr>
<tr>
<td>Nisin</td>
<td>Rosemary extract</td>
<td>1.63 ± 0.02</td>
<td>nd</td>
<td>0.63 ± 0.01</td>
<td>11.62 ± 0.86</td>
</tr>
<tr>
<td>Nisin</td>
<td>Ascorbic acid</td>
<td>2.12 ± 0.14</td>
<td>9.09 ± 0.38</td>
<td>0.99 ± 0.31</td>
<td>2.39 ± 0.37</td>
</tr>
<tr>
<td>Nisin</td>
<td>No antioxidant</td>
<td>2.14 ± 0.13</td>
<td>9.39 ± 0.06</td>
<td>0.89 ± 0.13</td>
<td>2.00 ± 0.46</td>
</tr>
<tr>
<td>Untreated cells</td>
<td>Propyl gallate</td>
<td>1.76 ± 0.33</td>
<td>9.37 ± 0.58</td>
<td>0.49 ± 0.01</td>
<td>5.13 ± 3.43</td>
</tr>
<tr>
<td>Untreated cells</td>
<td>BHT</td>
<td>1.88 ± 0.19</td>
<td>nd</td>
<td>0.62 ± 0.08</td>
<td>5.74 ± 0.45</td>
</tr>
<tr>
<td>Untreated cells</td>
<td>Rosemary extract</td>
<td>1.54 ± 0.22</td>
<td>nd</td>
<td>0.66 ± 0.00</td>
<td>12.11 ± 0.37</td>
</tr>
<tr>
<td>Untreated cells</td>
<td>Ascorbic acid</td>
<td>1.72 ± 0.33</td>
<td>9.31 ± 0.15</td>
<td>0.85 ± 0.01</td>
<td>1.66 ± 1.49</td>
</tr>
<tr>
<td>Untreated cells</td>
<td>No antioxidant</td>
<td>1.72 ± 0.34</td>
<td>9.24 ± 0.08</td>
<td>0.93 ± 0.07</td>
<td>0.93 ± 0.56</td>
</tr>
</tbody>
</table>

Nd: Not determined
Figure 5.7 Growth of *L. monocytogenes* in BHI broth at 30°C. Antioxidant treated cells transferred to nisin (200 IU/ml) (*), Antioxidant treated cells transferred to BHI broth (▲), Healthy cells transferred to nisin (○), No addition (●). Addition of antioxidant at 0 h and transfer to nisin at 3 h. Each point represents the mean ± standard deviation of two independent experiments tested in duplicate. Arrows mark undetectable counts (<10 CFU/ml).
Table 5.5 Growth parameters for *L. monocytogenes* NCTC5105 incubated at 30°C in the presence of nisin following treatment with antioxidants. Values represent the mean ± standard deviation of two independent experiments tested in duplicate.

<table>
<thead>
<tr>
<th>1st Treatment</th>
<th>2nd Treatment</th>
<th>No (log cfu ml⁻¹)</th>
<th>Nmax (log cfu ml⁻¹)</th>
<th>μmax (h⁻¹)</th>
<th>t-lag (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propyl gallate</td>
<td>Nisin</td>
<td>1.07 ± 0.07</td>
<td>8.65 ± 0.07</td>
<td>0.69 ± 0.01</td>
<td>2.91 ± 0.19</td>
</tr>
<tr>
<td>BHT</td>
<td>Nisin</td>
<td>2.72 ± 0.18</td>
<td>Nd</td>
<td>0.68 ± 0.01</td>
<td>4.85 ± 0.72</td>
</tr>
<tr>
<td>Rosemary extract</td>
<td>Nisin</td>
<td>nd</td>
<td>Nd</td>
<td>nd</td>
<td>Nd</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>Nisin</td>
<td>2.54 ± 0.13</td>
<td>Nd</td>
<td>0.67 ± 0.01</td>
<td>2.09 ± 0.25</td>
</tr>
<tr>
<td>No antioxidant</td>
<td>Nisin</td>
<td>1.29 ± 0.05</td>
<td>Nd</td>
<td>0.68 ± 0.02</td>
<td>0.85 ± 0.07</td>
</tr>
<tr>
<td>Untreated cells</td>
<td>Propyl gallate</td>
<td>4.79 ± 0.02</td>
<td>nd</td>
<td>1.06 ± 0.04</td>
<td>1.16 ± 0.13</td>
</tr>
<tr>
<td>Untreated cells</td>
<td>BHT</td>
<td>4.66 ± 0.03</td>
<td>9.06 ± 0.01</td>
<td>1.07 ± 0.04</td>
<td>0.85 ± 0.23</td>
</tr>
<tr>
<td>Untreated cells</td>
<td>Rosemary extract</td>
<td>4.00 ± 0.03</td>
<td>8.83 ± 0.49</td>
<td>1.02 ± 0.98</td>
<td>2.38 ± 0.27</td>
</tr>
<tr>
<td>Untreated cells</td>
<td>Ascorbic acid</td>
<td>5.13 ± 0.10</td>
<td>9.19 ± 0.01</td>
<td>0.96 ± 0.01</td>
<td>0.26 ± 0.26</td>
</tr>
<tr>
<td>Untreated cells</td>
<td>No antioxidant</td>
<td>5.15 ± 0.07</td>
<td>9.31 ± 0.03</td>
<td>1.03 ± 0.01</td>
<td>0.44 ± 0.15</td>
</tr>
</tbody>
</table>

Nd: Not calculated due to undetectable viable counts
Comparison of the three modes of treatment with nisin and antioxidants clearly indicates that the synergistic effect is observed only when both components (nisin and antioxidants) are present simultaneously (Fig. 5.8).

**Figure 5.8** Levels of *L. monocytogenes* after 24h of incubation at 30°C under different treatments of nisin and/or antioxidants (AO). A: Simultaneous addition of nisin + AO, B: Initial addition of nisin and subsequent transfer to AO, C: Initial addition of AO and subsequent transfer to nisin, D: Nisin alone, E: AO alone. Each point represents the mean ± standard deviation of two independent experiments.
5.3.8 Synergistic effect of nisin and antioxidants at low temperatures

The observed synergistic effect of nisin with propyl gallate, rosemary extract and BHT against *L. monocytogenes* NCTC5105 was also investigated at 8°C. The addition of 200 ppm propyl gallate or 750 ppm rosemary extract in BHI broth containing 200 IU nisin /ml exhibited a remarkable effect, as over a 28 day period no growth (< 1log CFU ml⁻¹) was detected after the initial reduction in *Listeria* counts (Fig 5.9).

The calculated mean values for specific growth rate (μmax) and lag time (t-lag) using Microfit software showed that the combination of nisin with BHT (μmax = 0.84 d⁻¹, t-lag = 5.16 days) was able to delay growth more effectively than nisin alone (μmax = 1.56 d⁻¹, t-lag = 4.22 days) but still after 21 days of storage counts exceeded 7 log CFU ml⁻¹ (Fig 5.9, Table 5.9).

Addition of each antioxidant alone did not produce initial count reduction. Propyl gallate and rosemary extract produced longer lag phases (2.07 and 8.09 days respectively) and lower mean growth rates (0.39 d⁻¹ and 0.79 d⁻¹ respectively), indicating a slight bacteriostatic effect on *L. monocytogenes* (Table 5.6). Exposure to BHT alone had no effect on growth, with counts being almost identical to that of the control (Fig. 5.9).
Figure 5.9 Growth of *L. monocytogenes* NCTC5105 incubated at 8°C under conditions of simultaneous exposure to nisin (200 IU/ml) and different antioxidants; Propyl gallate (1 mM), BHT (1 mM), and rosemary extract (750 ppm). Values represent the mean ± Standard deviation of two independent experiments tested duplicate. Arrows mark undetectable counts (<10 CFU/ml).
Table 5.6 Growth parameters for *L. monocytogenes* NCTC5105 incubated at 8°C under conditions of simultaneous exposure to nisin (200 IU/ml) and different antioxidants; Propyl gallate (1 mM), BHT (1 mM), and rosemary extract (750 ppm). Values represent the mean ± standard deviation of two independent experiments tested duplicate.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial log reduction</th>
<th>No (log cfu ml⁻¹)</th>
<th>Nmax (log cfu ml⁻¹)</th>
<th>μmax (days⁻¹)</th>
<th>t-lag (days)</th>
<th>t-d (days)</th>
<th>Days to Nmax</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisin + Propyl gallate</td>
<td>3.07</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>Nd</td>
<td>Nd</td>
<td>nd</td>
<td>&gt;28</td>
</tr>
<tr>
<td>Nisin + BHT</td>
<td>2.6</td>
<td>1.83 ± 0.55</td>
<td>8.17 ± 0.56</td>
<td>0.84 ± 0.16</td>
<td>5.16 ± 2.67</td>
<td>0.83 ± 0.14</td>
<td>21</td>
</tr>
<tr>
<td>Nisin + Rosemary extract</td>
<td>2.4</td>
<td>2.61</td>
<td>&lt;10</td>
<td>Nd</td>
<td>nd</td>
<td>nd</td>
<td>&gt;28</td>
</tr>
<tr>
<td>Nisin alone</td>
<td>2.7</td>
<td>2.09 ± 0.22</td>
<td>8.02 ± 0.24</td>
<td>1.56 ± 0.22</td>
<td>4.42 ± 0.67</td>
<td>0.44 ± 0.10</td>
<td>28</td>
</tr>
<tr>
<td>Propyl gallate</td>
<td>NR</td>
<td>4.55 ± 0.12</td>
<td>8.51 ± 0.20</td>
<td>0.39 ± 0.04</td>
<td>2.07 ± 1.44</td>
<td>1.78 ± 0.06</td>
<td>28</td>
</tr>
<tr>
<td>BHT</td>
<td>NR</td>
<td>4.71 ± 0.50</td>
<td>9.28 ± 0.56</td>
<td>0.99 ± 0.29</td>
<td>0.8 ± 2.26</td>
<td>0.70 ± 0.20</td>
<td>14</td>
</tr>
<tr>
<td>Rosemary extract</td>
<td>NR</td>
<td>4.25 ± 0.04</td>
<td>8.99 ± 0.05</td>
<td>0.79 ± 0.03</td>
<td>8.09 ± 0.34</td>
<td>0.87 ± 0.02</td>
<td>28</td>
</tr>
<tr>
<td>No addition</td>
<td>NR</td>
<td>4.75 ± 0.09</td>
<td>9.36 ± 0.06</td>
<td>1.58 ± 0.11</td>
<td>0.75 ± 0.30</td>
<td>0.44 ± 0.05</td>
<td>10</td>
</tr>
</tbody>
</table>

NR: no reduction in viable counts
Nd: Not calculated due to undetectable viable counts
5.3.9 Synergistic effect of nisin and propyl gallate in UHT milk

The simultaneous exposure to nisin and propyl gallate in whole and skimmed milk was evaluated by plate count enumeration over a period of 35 days at 8°C. From Figure 5.10 it is apparent that their combination in skimmed milk caused significant inhibition of growth. Due to the presence of propyl gallate the injury caused by nisin was more extensive rendering cells incapable for recovery. Hence, at the end of storage period counts remained below detection levels (< 1log CFU ml⁻¹).

Nisin on its own produced considerable population reduction, but gradually injured cells were repaired and started growing at lower growth rates. The use of antioxidants alone did not produce any bactericidal effects but delayed growth as shown in Table 5.7.

A different profile was seen in whole milk. No cell reduction was observed when nisin was used on its own or combined with the antioxidant. The pattern of growth for the nisin alone resembled that of the control, whereas for the combination with propyl gallate, growth was very similar to that of propyl gallate added on its own.

Figure 5.10 Rate of *L. monocytogenes* growth in UHT milk at 8°C using combinations of nisin and/or propyl gallate (PG). Values represent the mean ± standard deviation of two independent experiments tested in duplicate.
Table 5.7 Growth parameters for *L. monocytogenes* NCTC5105 in UHT milk stored at 8°C under conditions of simultaneous exposure to nisin and propyl gallate (1 mM). Values represent the mean ± standard deviation of two independent experiments tested in duplicate.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No (log cfu ml⁻¹)</th>
<th>Nmax (log cfu ml⁻¹)</th>
<th>µmax (days⁻¹)</th>
<th>t-lag (days)</th>
<th>t-d (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisin + Propyl gallate (WM)</td>
<td>4.44 ± 0.12</td>
<td>7.06 ± 0.31</td>
<td>0.41 ± 0.10</td>
<td>nd</td>
<td>1.69 ± 0.17</td>
</tr>
<tr>
<td>Nisin (WM)</td>
<td>4.41 ± 0.15</td>
<td>7.88 ± 0.13</td>
<td>0.62 ± 1.57</td>
<td>nd</td>
<td>1.11 ± 0.11</td>
</tr>
<tr>
<td>Propyl gallate (WM)</td>
<td>4.41 ± 0.10</td>
<td>7.58 ± 0.38</td>
<td>0.38 ± 0.07</td>
<td>nd</td>
<td>1.84 ± 0.14</td>
</tr>
<tr>
<td>Propyl gallate (SM)</td>
<td>4.41 ± 0.10</td>
<td>7.66 ± 0.40</td>
<td>0.40 ± 0.07</td>
<td>nd</td>
<td>1.72 ± 0.13</td>
</tr>
<tr>
<td>No addition (WM)</td>
<td>4.41 ± 0.28</td>
<td>7.70 ± 0.14</td>
<td>2.81 ± 3.52</td>
<td>0.33 ± 2.10</td>
<td>Nd</td>
</tr>
</tbody>
</table>

Nd: not determined
5.3.10 Interaction of nisin with lipids

The total loss of nisin activity in whole fat UHT milk was further investigated. The fate of nisin was studied by determining the increase in the nisin minimum inhibitory concentration against *L. monocytogenes* in media containing vegetable oil and by assessing the partition of nisin between an aqueous and vegetable oil phase.

As seen in Figure 5.11 the nisin MIC after 24 hours of incubation at 30°C appears to be elevated in the presence of oil. The observed increase highly correlated with the oil concentrations, 500-750 IU/ml for the 3:1 (water:oil) solutions and 1000-1500 IU/ml for the 1:1 (water-oil) solutions. Incubation for a further 24 hours increased the MIC for all test and control samples to the next level of nisin.

Nisin activity was also estimated with the bioassay method against *M. luteus*. Results showed in two separate experiments that after the process of emulsification there was approximately a three-fold reduction of the initially detected levels for the 3:1 (water:oil) mixture of nisin and oil solution and approximately 90% nisin loss for the 1:1 (water:oil) mixture (Table 5.8).

Both methods exhibited similar patterns for nisin behaviour in the presence of oil. Nisin appears to act as emulsifying agent (Jaber et al., 2000) in this kind of mixtures, losing its biological activity against microbes.
Figure 5.11 Nisin MICs against *L. monocytogenes* NCTC5105 in 3:1 and 1:1 nisin containing water-oil mixtures after 24 h (□) and 48 h (■). (Number of replicas=9)

Table 5.8 Water extractable nisin in oil and nisin (100 IU/ml) solution before and after emulsification.

<table>
<thead>
<tr>
<th>Type of mixture</th>
<th>Nisin content (IU/ml)</th>
<th>% Recovery</th>
<th>Nisin content (IU/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>3:1 Mixture</td>
<td>93</td>
<td>100</td>
<td>28</td>
<td>30.10</td>
</tr>
<tr>
<td>1:1 Mixture</td>
<td>105</td>
<td>100</td>
<td>11</td>
<td>10.40</td>
</tr>
</tbody>
</table>
5.4 DISCUSSION

The antioxidants used in this study showed no bactericidal effect, but they did cause retardation of growth by decreasing growth and increasing lag phase (Table 5.3). Cells exposed to antioxidants did not recover from an initial shock and then grew normally, but their growth was subject to a continued drag consistent with an energy burden imposed on the cells by the presence of antioxidant, as indicated by the slower growth rate once growth resumed. This can be expected in view of the lipophilic nature of phenolic compounds which allows them to incorporate easily into the cell membrane, disrupting the function of various enzymes, especially components of energy-converting systems such as electron transport chains (ETCs) and ATPases embedded in the lipid bilayer (Lambert et al., 2001; Kubo et al., 2002). This could explain the fact that the antimicrobial action of many antioxidants is due to their ability to inhibit respiration. The extent of damage and whether this could cause cell death is a function of both the compound concentration and the initial microbial load (Yousef et al., 1991; Oluyemi et al., 1996). Since no bactericidal effect was observed in this study, it was assumed that all compounds were added at sub-lethal concentrations. Indeed, previous studies have shown that microbial inhibition occurred at higher concentrations than the ones used in our experiments (Yousef et al., 1991; Oluyemi et al., 1996; Kubo et al., 2002).

Nisin is known to kill bacteria by targeting the cell membrane through pore formation (Breukink and Kruijff, 1999). Pore formation causes rapid efflux of small cytoplasmic compounds, collapse of vital ion gradients and dissipation of the membrane proton motive force, resulting in cell death (Breukink and de Kruijff, 1999). The C-terminus of the polypeptide seems to play an important role in the binding of nisin to the target membrane due to its positive charge, whereas the amphiphilic properties of the peptide allow it to insert into the lipid phase of the membrane. Hence, cell surface hydrophobicity, differences in the phospholipid concentration and changes in that fatty acid composition of the cytoplasmic membranes can produce variations in nisin activity (Ming and Daeschel, 1995; Davies et al., 1996). In this context, synergism of nisin with other antimicrobial agents has been attributed to cell membrane destabilisation induced by them, making bacteria more susceptible to the action of nisin (Thomas et al., 1998; Ettayebi et al., 2000; Singh et al., 2001; Nattress et al., 2001; Yamazaki et al., 2004).
However, this study shows that the synergistic effect between nisin and certain antioxidants was not due to an increase in the lethal effect of nisin in the presence of the antioxidant as a result of the antioxidant causing a loss of membrane integrity. When cells were exposed to nisin plus antioxidant recovery was delayed, and when growth resumed, the growth rate was the same as when untreated cells were placed in the antioxidant. This indicates that cells not killed by nisin were subject to injury, the repair of which is energy dependent. ATP synthesis needed for cell repair (Ray et al., 1986) requires high amounts of energy generated by the cell, hence, since antioxidants can interfere or disrupt to some extent energy-converting systems recovery of injured cells is delayed by their presence.

The role of antioxidants in delaying recovery from nisin injury and thus enhancing its activity is supported by the results of sequential exposure to nisin and antioxidants. When suspensions were exposed to antioxidants following nisin treatment, their recovery was delayed in comparison with non-treated cells. However, when nisin treatment followed exposure to antioxidants the recovery was similar, whether or not the cells had been exposed to antioxidants. The only exception to this was rosemary extract, but this can be due to the greater antimicrobial effect of the particular compound, which was used at concentration approaching its MIC.

At lower temperatures the combination of nisin with antioxidants exhibited a similar synergistic effect to that seen for 30°C. The presence of propyl gallate produced an immediate stimulation of nisin's bactericidal effect with survivors estimated below detectable levels throughout storage. With rosemary extract plus nisin, the initial cell reduction was less than in the nisin control but counts continued to decline with time and after 28 days no viable cells could be detected. This implies that the mechanism of action for the two antioxidants may differ from each other and from that of other antioxidants. L. monocytogenes is able to survive at low temperatures by increasing the proportion of the unsaturated fatty acyl chains of lipids (Abee et al., 1994), which could facilitate the incorporation of lipophilic compounds such as rosemary extract and propyl gallate. Hence, under these conditions, impairment of cellular membrane function can be more extensive and can stimulate the bactericidal effect of nisin. Antioxidants have also been reported to induce reactive oxygen species (ROS) generation (Kubo et al., 2002; Zanicchelli et al., 2005). Membrane lipids are abundant in unsaturated fatty acids, and their oxidation leads to decrease in the membrane fluidity and disruption of structure and
function. Hence, the ROS generation and modification of membrane composition as an adaptation response to low temperatures may give another explanation for the enhanced effect of the combination of nisin with antioxidants.

The antioxidant chosen for the test in a food system (milk) was propyl gallate, since it was found to be the most effective antioxidant when combined with nisin in laboratory media. Propyl gallate on its own had no bactericidal effect, but retarded growth compared with control. Calculation of growth parameters for propyl gallate in both skimmed and whole milk and comparison with those obtained for laboratory media were shown to be similar, indicating that in contrast with previous studies (Pandit and Shelef, 1994; Skandanis and Nychas, 2000) the composition of the food system had no impact upon the efficacy of this particular antioxidant.

The combination of nisin with propyl gallate totally suppressed growth of the test strain in skimmed milk over a period of 35 days and this is in good agreement with the findings obtained for the same combination in BHI broth at 8°C. However, this was not seen in whole milk and that was taken as evidence for nisin interactions with fat. Bacterial sensitivity to nisin in milk has been previously found to be strongly dependent on milk fat concentration (Jung et al., 1992) with activity decreasing at increased fat levels or totally inhibited in whole milk (Jones, 1974). However there have been cases where fat content had no effect on nisin activity in extending the shelf life of some dairy products (Phillips et al., 1983; Wirjantoro et al., 2000).

Increase in the nisin minimum inhibitory concentration against *L. monocytogenes* and reduced nisin recovery from oil-in-water system substantiated further the poor antimicrobial protection of nisin in food systems with high fat content. Our hypothesis is that most of the nisin is bound around a great number of water droplets dispersed in the oil as suggested by Jaber et al., (2000) or is likely to adhere to the interface between oil and water. The basis of this phenomenon was not investigated in this study and provides an area for future work.

Overall, antioxidants can act synergistically with nisin to inhibit growth of *L. monocytogenes* especially at low temperatures. Retardation in recovery and growth of nisin-injured cells through disruption of cellular respiratory and energy functions is a
possible mechanism. The consistent effectiveness of propyl gallate and nisin against 
*L. monocytogenes* in skimmed milk gives the basis for the incorporation of these 
compounds in numerous processed food products, with particular emphasis to low fat 
systems as fat was shown to hinder significantly nisin’s activity.
CHAPTER 6

GENERAL DISCUSSION AND FUTURE WORK
CHAPTER 6

GENERAL DISCUSSION AND FUTURE WORK

Public concern over possible health hazards posed by the use of synthetic preservatives and the notion that heavily processed products are of poor nutrient content have focused the interest of food scientists and technologists towards foods that have received mild preservation treatments and which are free from chemical preservatives. Among alternative preservation methods, the incorporation of nisin has received considerable attention in recent years and it has found numerous applications in different products worldwide. The use of nisin in meat systems as a partial replacement of nitrites and to reduce thermal processing has been widely investigated. However, nisin demonstrates variable effectiveness in meat products. Recent reports have noted the importance of interactions of nisin with antagonistic compounds such as meat components. This will act as a constraint on the further use of nisin. Therefore, it is essential to determine which factors antagonize nisin activity, the extent of this inhibition and the mechanisms involved with a view to finding solutions to abolish or reduce this inhibition of nisin activity.

For the practical application of nisin, awareness of the biologically active nisin content and the rate of its loss in food matrices are essential to estimate the shelf life and microbiological safety of products, as loss of nisin activity below a threshold inhibitory level may be ultimately responsible for growth of spoilage or pathogenic bacteria (Montville et al., 1995). Nisin recovery immediately after its addition to the food matrix is dependent on the extraction method used. The levels of nisin extracted using water alone are significantly lower than the levels obtained after acid/heat extraction (Present study, Aasen et al., 2003). Boiling meat at acidic pH, breaks down ionic bonds, liberating protein-bound nisin. Hence the better recovery seen with heat-acid extraction indicate that a significantly high proportion of residual nisin is adsorbed onto the abundant muscle proteins in meat. (Bell and De Lacy, 1986; Coventry et al., 1995; Davies et al., 1999). Whether or not protein-bound nisin is biologically active is a subject slightly touched in the present thesis (Appendix 2), where the antimicrobial activity of nisin against B. cereus was tested in the presence of model proteins such as casein, bovine albumin and
protamine. However, this topic undoubtedly needs to be investigated further and provides an interesting area for future work.

A more rapid drop in total nisin content (heat/acid extractable) compared to that of free nisin (water-extractable) was seen for both ground meat and meat extracts. This may be due to bound nisin acting as a reservoir of nisin replenishing free nisin levels, but this possibility was not examined in this thesis and needs to be assessed in the future.

Loss of nisin activity in meat has been partly ascribed to the formation of a nisin-glutathione adduct. Glutathione is found in mammalian cells, plants and microorganisms, and at levels of 156 – 627 nmol/g wet weight in beef, chicken and pork (Wierzbicka et al., 1989). The free sulfhydryl group of GSH has been proposed as the binding site for nisin in fresh meat (Rose et al, 1999). Its reaction with the dehydro amino acid residues of nisin yields GSH S-conjugates and this has been found to be partially mediated by a glutathione S-transferase (GST) which constitutes about 0.3% of the total soluble protein in bovine muscle (Rose et al, 2002). Activity is lost more quickly in raw meat than in cooked meat and this has been taken as evidence that the reaction is enzyme-mediated. In the work reported here, formation of the nisin-glutathione adduct has been confirmed but has been shown not to be enzyme mediated. Glutathione has been reported to reduce intramolecular disulfide bonds to sulfhydryl groups (Jocelyn, 1972; Haschemeyer and Haschemeyer, 1973). Reduction of disulfide bonds promotes the interaction of proteins and leads to gel formation through disulfide linkages (Hayakawa and Nakamura, 1986; Li-Chan and Nakai, 1991). Thus, retention of activity in cooked meat is due to loss of free sulfhydryl groups during cooking as a result of the reaction of glutathione with proteins and not the result of differences in the microbial load in raw and cooked meat or the loss of endogenous enzyme activity on cooking.

The better recovery of nisin seen in heat-processed meat extract where nisin addition followed thermal treatment compared to heat-processed meat extract where nisin is added before pasteurisation may be explained not only by the loss of free glutathione, but to inactivation of other free SH- groups of meat proteins, which could be potential sites for nisin binding. On the other hand, heating nisin with meat may have the advantage of reducing the numbers of Gram-negative bacteria present (Boziaris et al., 1998). Therefore
the balance between the benefits and drawbacks of adding nisin prior to or after thermal processing will depend on the product type and the nature of the microflora of concern.

Although the inactivation of glutathione and protein sulfhydryl groups in meat results in better efficacy of nisin in cooked meat, its use in raw meat products is still problematic since these components remain available to interact with nisin. To extend the use of nisin in raw meat products, it is imperative to abolish their restraining effect on its activity. In dough mixing, dehydroascorbic acid promotes the oxidation of sulfhydryl groups in gluten proteins to form disulfide bonds between protein subunits. The possibility that dehydroascorbic acid will act the same way in meat, thereby protecting nisin, was investigated. The introduction of ascorbic and dehydroascorbic acid into nisin-containing laboratory media with added glutathione, or into meat model systems where free thiol groups are naturally present, is able to remove SH-group inhibitory effect and exert a protective effect on nisin activity (Present thesis). Although, their addition is successful in combating growth of *L. monocytogenes*, we found that this was not due to a direct effect of these compounds on glutathione, as hypothesized initially. The exact mechanism of the synergy seen has not been elucidated and provides an extended subject for future research.

Nevertheless, the use of these agents in meat products along with nisin is still a promising system which ensures both the microbiological safety of the product and technological stability. The direct addition of dehydroascorbic acid is not possible, as it is not a permitted additive. However, ascorbic acid is often added during curing of meats and its oxidation to dehydroascorbic acid by sodium nitrite and other heme-containing compounds contributes to colour improvement and stabilisation. Moreover, its presence in meat products can minimize nitrosamine formation, and the efficient synergy with nisin may be a feasible approach for the production of low nitrite cured products.

In foods like meat products, lipid oxidation is responsible for the reduction in nutritional quality as well as changes in flavour (Sallam *et al.*, 2004), while microbial contamination can bring major public health hazards and economic losses in terms of food poisoning and meat spoilage. These can be controlled or minimized by the use of synthetic or natural food additives. With respect to lipid deterioration, various antioxidants, especially those of plant origin possess antimicrobial properties but the inhibitory concentrations reported
exceed the levels used in manufacturing practice (Yousef et al., 1991; Oluyemi et al., 1996; Kubo et al., 2002). Moreover, the use of natural antioxidants in foods as preservatives is rather limited since effective antimicrobial doses may affect food flavour (Lambert et al., 2001). The antioxidants used in this study added at the higher acceptable/recommended levels have no lethal effect but delay growth by decreasing growth rate and extending lag phase, possibly because they divert energy from growth-related functions.

However, their application with nisin has a synergistic inhibitory effect against \textit{L. monocytogenes}; an effect seen mainly when both antioxidants and nisin are added simultaneously. In other studies the application of nisin with other antimicrobial compounds, such as thymol, carvacrol, lysozyme, garlic extract etc., showed that all these treatments produce a better lethal effect than nisin alone (Thomas et al., 1998; Ettayebi et al., 2000; Singh et al., 2001). Thus, extending nisin activity against Gram-negative bacteria (Stevens et al., 1991; Cutter and Siragusa, 1995a; Scannell et al., 1997; Boziaris and Adams, 1998; Ariyapitipun et al., 1999) or enhancing its activity against relatively resistant Gram-positive bacteria, like \textit{L. monocytogenes} (Davies and Adams, 1994) may be a promising solution to this problem.

Another practical aspect of applying nisin as part of a multifactorial preservation method is related to the fact that nisin is rather expensive, hence, the synergy between nisin and antioxidants, studied in this thesis, or with other additives would also mean that the effective concentrations of nisin could be lowered to achieve the desired antibacterial activity, thus reducing production costs.

The combination of nisin with certain antioxidants at refrigeration temperatures was shown to be more effective, with no detectable \textit{Listeria} counts after a month of storage. Antioxidants are used to prevent the deterioration of fatty food stuffs like milk products and ice-creams or comminuted meat products. Therefore the prospect of using them with nisin in products that are stored chilled looks promising in order to maintain product safety and possibly extend shelf life.

However, the food environment remains an important limiting factor. When the combination of nisin with propyl gallate in whole UHT milk was tested, a complete loss
of the nisin antimicrobial activity was seen (Present study; Jung et al., 1992; Chumchalova et al., 1998; Zapico et al., 1999) and this is consistent with higher inhibitory concentrations needed for the total inhibition of L. monocytogenes in laboratory media where vegetable oil was added. Nisin binding to the fatty components of foods, however, may be resolved with the use of emulsifiers, such as monolaurin (Bell and De Lacy, 1987), sucrose esters of fatty acids (Thomas et al., 1998) and diglycerol fatty acid esters (Yamazaki et al., 2004).

Nisin's amphiphilic properties are strongly related to its antimicrobial action, but its positive charge and the high content of hydrophobic amino acids make it vulnerable to interactions with other food macromolecules resulting in reduced efficacy. Taking into consideration the findings of this study, as well as those of other researchers, it is clear that nisin performs better when its use is supplemented with other preservation agents or methods. This further supports the view of Leistner and Gorris (1995), where food preservation by multiple preservatives in small amounts is superior to preservation with large amounts of a single preservative. The use of natural preservatives or mild preservation techniques is undeniably desired. However, most of the novel preservation techniques and natural antimicrobials still have high costs. Therefore at present, the use of natural antimicrobial agents like nisin can be feasible when used as an element of a multi-preservation method to partially replace synthetic compounds.
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APPENDIX 1

In this appendix the growth curve of *Listeria monocytogenes* NCTC5105 used in the experiments is shown.

Cells were grown in BHI broth incubated statically at 30°C. Growth was monitored both by the spread plate technique on BHI agar and by optical density measurement at 600 nm. The point where growth was visible by turbidity was also established to approximately 5 log CFU/ml (dashed line).

For all nisin experiment stationary phase cells were used.
APPENDIX 2

INVESTIGATION OF ANTIMICROBIAL ACTIVITY OF PROTEIN-BOUND NISIN

The effect of proteins on nisin activity was studied. Changes in the minimum Inhibitory concentrations (MIC) against \textit{B. cereus} 2599 were investigated in the presence of casein, albumin bovine and protamine sulphate.

BHI broths were prepared containing 0, 50, 100, 500 and 1000 µg/ml albumin bovine, protamine sulphate and casein, respectively. The pH was adjusted at 7. At this pH value albumin is believed to be charged negatively as it has a PI of about 4.7-4.9, protamine is charged positively (PI around 12) and casein is charged negatively.

Volume of 30 µl of nisin (500, 400, 350, 300, 250, 200, 150, 100 and 0 IU/ml) and 30µl of stationary phase grown \textit{B. cereus} 2599 were added to 240µl of protein-containing broths. Initial inoculum was estimated at $10^3$ CFU/ml. The minimal inhibitory concentration was determined as the level of nisin, where no increase in the optical density (OD) at 600 nm was observed.

As shown in Figures 1, 2, 3 all the proteins used, with the exception of protamine at 1000 ppm, had no effect on the nisin MIC against \textit{B. cereus} and that was seen for all concentrations tested. Small variations seen in the MIC may be due to experimental errors.

Assuming that nisin binds on proteins via electrostatic interactions, this experiment shows that protein-bound nisin may be still biologically active. However, no sound conclusions can be made and this needs to be explored further.
Figure 1 Determination of the nisin Minimum Inhibitory Concentration (MIC) against B. cereus 2599 at 30°C, pH 7 in the presence of casein. Control (-), 50 IU nisin/ml (○), 100 IU nisin/ml (●), 150 IU nisin/ml (△), 200 IU nisin/ml (♦), 250 IU nisin/ml (※), 300 IU nisin/ml (☆), 350 IU nisin/ml (△), 400 IU nisin/ml (●), 500 IU nisin/ml (♦).
**Figure 2** Determination of the nisin Minimum Inhibitory Concentration (MIC) against *B. cereus* 2599 at 30°C, pH 7 in the presence of casein. Control (+), 50 IU nisin/ml (-), 100 IU nisin/ml (-), 150 IU nisin/ml (+), 200 IU nisin/ml (•), 250 IU nisin/ml (•), 300 IU nisin/ml (X), 350 IU nisin/ml ( ), 400 IU nisin/ml ( ), 500 IU nisin/ml ( ).
Figure 3: Determination of the nisin Minimum Inhibitory Concentration (MIC) against B. cereus 2599 at 30°C, pH 7 in the presence of casein. Control (•), 50 IU nisin/ml (-), 100 IU nisin/ml (-), 150 IU nisin/ml (+), 200 IU nisin/ml (•), 250 IU nisin/ml (*), 300 IU nisin/ml (X), 350 IU nisin/ml (▲), 400 IU nisin/ml (●), 500 IU nisin/ml (★).