Bacterial Injury and Sensitisation of Gram-negatives to Nisin

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SUMMARY

Nisin is a bacteriocin produced by *Lactococcus lactis* subsp. *lactis*, which is active against Gram-positive organisms including bacterial spores. It is not generally active against Gram-negative bacteria, yeasts and fungi. Gram negatives show nisin-sensitivity when their outer membrane permeability is altered by various means, such as treatments with chelators, e.g. EDTA, osmotic shock, heating, freezing, freeze-drying, high-pressure etc.

Application of chelators and nisin is effective against Gram-negatives when exogenous nisin is added. Nisin produced *in situ* and chelators are not an effective combination, since nisin production follows the pH drop caused by sugar fermentation, and this interferes with the sequestering ability of the chelators. Presence of nisin during thermal inactivation of Gram-negatives though is effective. Bacteria become structurally injured during heating showing sensitivity to agents like SDS and deoxycholate and extended detection times by impedimetry. These injured bacteria are inactivated by nisin, with a concomitant reduction of the measured D-values. Low pH and the presence of small amount of chelators enhance the injury and inactivation and reduce D-values further. Gram-negative bacteria injured by chilling and freezing are also sensitive to nisin.

The effectiveness of nisin is reduced in a food environment mostly of nisin binding to fat, and food particles. D-values were decreased less or not at all in egg white and liquid whole egg, respectively, and rapid chilling of bacteria attached to chicken skin in presence of nisin did not give the effect seen in laboratory media.

Nisin is active against heat-, chill-, and freezing-stressed Gram-negatives only if it is present during the treatments. When the stress factor is removed, the bacteria recover their nisin resistance, implying transient susceptibility to nisin, but not to smaller molecules. This is probably due to rapid reorganisation and restoration of OM permeability damage, rather than biochemical repair.

The LPS chain length influences the sensitisation of Gram-negatives to nisin, only in the case of freezing, where the strain with the shorter LPS chain was more sensitive than the
wild type. Heat-, and freezing-stressed bacteria lost lipopolysaccharides and increased their cell surface hydrophobicity. This was not seen with chill-stressed bacteria, which were sensitive to nisin though. This indicates that release of LPS is not a prerequisite for nisin sensitivity in Gram-negatives.
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List of Abbreviations

$A_{400}$: absorbance at 400 nm
CM: Cell Membrane
CSH: Cell Surface Hydrophobicity
EW: Egg White
GLC: Gas Liquid Chromatography
HIC: Hydrophobic Interaction Chromatography
LPS: Lipopolysaccharide
LWE: Liquid Whole Egg
MATH: Microbial Adhesion To Hydrocarbons
MRD: Maximum Recovery Diluent
NA: Nutrient Agar
NA/SDS: Nutrient Agar supplemented with SDS
NB: Nutrient Broth
NPN: 1-N-phenylnaphthylamine
OM: Outer Membrane
PBS: Phosphate Buffered Saline
SDS: Sodium Dodecyl Sulfate
XLDA: Xylose Lysine Deoxycholate Agar
R.O.: Reverse Osmosis
OD: Optical Density
ATCC: American Type Culture Collection
USCC: University of Surrey Culture Collection
NCFB: National Collection of Food Bacteria
NCIB: National Collection of Industrial Bacteria
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CHAPTER 1

GENERAL INTRODUCTION
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1.1 NISIN

Nisin is a bacteriocin produced by certain strains of *Lactococcus lactis* subsp. *lactis* during fermentation (Adams and Moss 1995). Despite bacteriocins generally displaying inhibitory activity against closely related species (de Vos et al., 1991), nisin is active against a range of Gram positive bacteria, including spores (Delves-Broughton et al., 1996).

1.1.1 HISTORY

Nisin was first discovered in the 1920s, when problems arose during cheese making. Batches of milk starter culture used in the process had become contaminated with a nisin producing strain of *Lactococcus lactis* (then called *Streptococcus lactis*) and the development of cheese was detrimentally affected (Delves-Broughton, 1990). Nisin was named accordingly from group N (streptococci) Inhibitory Substance (Mattick and Hirsch, 1947). Initially, nisin was considered as a therapeutic agent in medical and veterinary science. Due to its rapid breakdown by digestive proteases, instability at physiological pH and its restricted antibacterial spectrum, it was found to be unsuitable for such purposes (Delves-Broughton and Gasson, 1994).

The potential use of nisin as a food preservative was first investigated by Hirsch in 1951, who demonstrated that clostridial gas formation in cheese could be prevented with the use of nisin-producing starter cultures (Hirsch et al., 1951). MacClintock et al., (1952) suggested that nisin was most effective in preventing clostridial spoilage in pasteurized processed cheese. Subsequently, numerous other applications were identified, and in
1969, nisin was approved for use as an antimicrobial in food by a Joint Food and Agriculture Organisation/World Health Organisation Committee (Delves-Broughton, 1990).

Nisin is sold as a commercial preparation with high and consistent activity under the trade name of Nisaplin (contains 2.5% nisin A) by Aplin and Barrett Ltd (Beaminster, Dorset, UK). Nisin is currently permitted as a food additive (E234) in over 50 countries including the EU and USA (Delves-Broughton et al., 1996).

1.1.2 STRUCTURE

Nisin belongs to a group of antimicrobial peptides or bacteriocins known as lantibiotics, which are produced by various Gram-positive bacteria, like Staphylococcus, Lactococcus, Streptococcus, Bacillus and Streptomyces (Jung 1991). Lantibiotics are relatively small peptides (19-34 amino acids) and are named as such because all contain the same unusual amino acids, lanthionine and/or \( \beta \)-methyl-lanthionine, which form intrachain thioether bridges (de Vos et al., 1991). Other unusual amino acids present are dehydroalanine (Dha) and dehydrobutyryne (Dhb).

Nisin’s complete structure was elucidated by Gross and Morell (1971). It is composed of 34 amino acids, contains two dehydroalanine (Dha), one dehydrobutyryne (Dhb), one lanthionine and four \( \beta \)-methyl-lanthionine residues, and has a molecular weight of 3354 Daltons (Figure 1.1.1). Dha and Dhb arise from dehydration of serine and threonine respectively, while condensation of Dha or Dhb with cysteine generates thio-ether bonds and the amino acids lanthionine and \( \beta \)-methyl-lanthionine, respectively (Ingram, 1970, Figure 1.1.2). Proton Nuclear Magnetic Resonance (\(^1\)H NMR) analysis and computer simulation of nisin show that it lacks regular secondary structure but exists in a rigid three-dimensional conformation due to the constraints imposed by the five thio-ether rings (Harris et al., 1992).
Figure 1.1.1 Structure of nisin. Dha dehydroalanine; Dhb, dehydrobutyryne; Ala-S-Ala, lanthionine; Aba-S-Ala, β-methyllanthionine. (Based on Gross and Morell 1971)

Figure 1.1.2 Mechanism for the synthesis of unusual amino acids found in nisin. (From Ingram, 1970)
Five polypeptides (nisins A to E) have been resolved from nisin preparations by countercurrent distribution between solvents. The preparation contained 49% nisin A, 32% B, 12% C and 3% of E (Berridge et al., 1952). Nisin B to E are thought to be degradation products of nisin A (Harris et al., 1992). ^1H NMR analysis of nisin A degradation products shows that nisin_{1-32} is nisin B and is as active as nisin_{1-34} (Chan et al., 1989; Shiba et al., 1986). Nisin fragments derived by reaction with cyanogen bromide have varying antimicrobial activity. The specific activity of nisin_{1-21} is 10% less than the intact nisin (Shiba et al., 1986; Liu and Hansen, 1990). Liu and Hansen reported that nisin_{22-34} has 90% reduced activity in contrast to Shiba et al., (1986), who reported that the fragment is inactive. When the third ring of nisin_{1-21} opens at Met_{17} or when the ring is removed by trypsin cleavage (nisin_{1-12}) the activity is drastically reduced (Shiba et al., 1986). These results show the importance of the rings, especially the third one, on antimicrobial activity of nisin. A natural nisin variant, nisin Z, has been described that has a substitution of His_{27} for Asn_{27} (Mulders et al., 1991).

1.1.3 PHYSICAL AND CHEMICAL PROPERTIES OF NISIN

The nisin molecule exhibits greatest stability under acidic conditions and is inactivated at alkaline pH (Hurst 1981). In solution or aqueous suspensions, nisin retains its activity when heated at acid pH values. In 1964, Tramer reported that nisin was stable to autoclaving at 115.6°C at pH 2 but 40% of its activity was lost at pH 5 and more than 90% was lost at 6.8 (Delves-Broughton et al., 1996). Pasteurisation temperatures are less damaging to nisin and at least 80% activity will be retained, for example, during standard processed cheese manufacture (Delves-Broughton et al., 1996). Large molecules such as those present in milk or broth have a protective effect (Delves-Broughton, 1990), so the degree of inactivation is less in foods than in buffers, but meat particles and fatty materials like phospholipids diminish the activity of nisin (Scott and Taylor, 1981a; Jung et al., 1992).

Nisin is more soluble at lower pH and becomes progressively less soluble as the pH approaches neutrality. At alkaline pH, nisin is practically insoluble. At pH 2.2 the
solubility is around 56 mg/ml, at pH 5 it is 3 mg/ml, and at pH 11 it is 1 mg/ml (Liu and Hansen, 1990). In practical food preservation, the levels of nisin are unlikely to exceed 0.25 mg/ml and solubility is never a problem (Delves-Broughton et al., 1996).

Nisin contains no aromatic amino acids and so has no absorbance at 260 or 280 nm. It is a basic polypeptide with isoelectric point in the alkaline range (Hurst 1981). Hence, at neutral and acidic pH, nisin has a net positive charge and lanthionine is known to introduce a high level of hydrophobicity to the molecule (Kaletta et al., 1989). According to Liu and Hansen (1990), nisin can form dimers or even oligomers, which possibly arise through a reaction between dehydroamino acids and amino groups of two or more nisin molecules.

1.1.4 ASSAY OF NISIN

The activity or potency of a nisin preparation is expressed in terms of International Units (IU), where 1 g of pure nisin is equivalent to 50x10^5 IU and 1 g of Nisaplin is equivalent to 1x10^6 IU (Aplin and Barrett Ltd, Dorset, UK).

A number of methods have been devised for the assay of nisin. In 1934 Cox recommended the use of methylene blue reduction for the detection of 'inhibitory streptococci' in milk (Hurst and Hoover, 1993). According to this test, starter organisms in milk rapidly reduce methylene blue to a colourless compound. If the milk contains nisin-producing streptococci, the time required for reduction is increased. Hirsch, in 1950, described not only a quantitative version of the methylene blue assay but also a more complex assay; the lag phase method. *Streptococcus agalactiae* was the test organism and within a range of 5-50 IU/ml the length of the lag phase was linearly related to the concentration of nisin (Hurst and Hoover, 1993).

In 1952 Berridge and Barrett developed the turbidimetric assay. The test organism, *L. cremoris*, was grown for 2.5-3 hours in broth containing different concentrations of nisin, the growth then being stopped with an organic mercurial disinfectant. A standard
was then plotted relating OD at 600 nm to nisin concentration (Hurst and Hoover, 1993).

The conventional horizontal agar diffusion technique, which is the method in current routine use for estimation of nisin, using the test organism Micrococcus flavus, subsequently named Micrococcus luteus (Fowler et al., 1975), was first described by Tramer and Fowler (1964). Nisin usually occurs in a polymeric form (MW>7000) and therefore, does not readily diffuse through agar. Tramer and Fowler used 1% Tween 20, to permit a simple diffusion assay of nisin. The size of the zone of inhibition is linearly related to nisin concentration, in the range of 0.5-10 IU/ml. This method is especially useful for the estimation of nisin in foods, because such samples may be cloudy or contain particulate matter. Tramer and Fowler also described the preparation of suitable controls to overcome problems, which interfere the assay such as binding of nisin or other inhibitors by food components.

New techniques such as measurements of released ATP from Lactobacillus casei following exposure to nisin (Waites and Ogden, 1987) and the ELISA method may well prove to be applicable (Falahee et al., 1990).

1.1.5 MODE OF ACTION

Like other cationic polypeptide antibacterial substances, the primary site of action of nisin is the bacterial membrane (Ruhr and Sahl, 1985). It was originally thought that nisin acted as a cationic surface-active detergent due to strong adsorption of nisin to cells causing leakage of ultraviolet-absorbing material and subsequent lysis (Ramseier, 1960). Reisenger et al., (1980) showed that nisin inhibits the murein synthesis. Henning et al., (1986) confirmed that observation but proved that inhibition of murein synthesis is not a primary mode of action.

It is now known that nisin acts as a membrane-depolarising agent, causing pore formation (Sahl et al., 1987; Gao et al., 1991; Garcera et al., 1993; Driessen et al.,
1995). Henning et al., (1986) showed that the antimicrobial effect of nisin is caused by an interaction of nisin with the phospholipid component of the cytoplasmic membrane. They demonstrated that various phospholipids and isolated cytoplasmic membrane fragments could antagonise the inhibitory effect of nisin, and that nisin will combine with phospholipids to form nisin-phospholipid complexes. Sohl et al., (1987) observed that nisin produced transient pores in planar lipid bilayers (black lipid membranes) in a voltage-dependent fashion. The pore formation required the existence of a sufficient trans-negative orientation membrane potential, (negative inside). Gao et al., (1991) in experiments with liposomes, showed that nisin activity requires a membrane potential or/and pH gradient (alkaline inside), and is influenced by the phospholipid composition. Further studies of Carcera et al., (1993) and Driessen et al., (1995) indicated that nisin disrupts the cytoplasmic membrane by incorporating into the membrane forming transient pores which are dependent upon proton motive force and membrane lipid composition. Driessen et al., (1995) and Moll et al., (1997) developed the 'wedge model' of nisin pore formation (Figure 1.1.3). The insertion of nisin into membranes which follows binding is not exclusively voltage-dependent since both components of proton motive force are by themselves sufficient for inducing its insertion giving rise to a wedge-like pore.

Nisin action against spores at the molecular level has been studied by Morris et al., (1984). They showed that nisin action against spores was caused by binding of nisin with sulphydryl groups on protein residues. Phospholipids were not implicated.

1.1.6 ANTIMICROBIAL PROPERTIES

Nisin shows antimicrobial activity against a wide range of Gram positive bacteria but shows little or no activity against Gram negative bacteria yeasts or moulds (Hurst 1981). However, three strains of Neisseria tested by Mattick and Hirsch (1947) were quite sensitive. Gram positive spore formers (Bacillus and Clostridium spp) are particularly sensitive to nisin, with spores being more sensitive than vegetative cells (Delves-Broughton et al., 1996). Generally the sensitivity of Gram positives varies. The closely allied Lactococcus lactis subsp. cremoris is the most sensitive, while faecal streptococci
Nisin binds to the anionic membrane surface leading to a high local concentration and disturbance of the lipid dynamics. Both components of the proton motive force (ΔpH, Δψ) are by themselves sufficient for inducing insertion of nisin into the membrane. Coinsertion of bound anionic phospholipid results in bending of the lipid surface, giving rise to a wedge-like pore.

Figure 1.1.3 Wedge model of nisin pore formation (Based on Moll et al., 1997).
like Enterococcus fae calculus are resistant (Hurst and Hoover, 1993). Nisin also shows activity against staphylococci and Listeria monocytogenes (Hurst and Hoover, 1993).

Nisin action against vegetative cells can be either bactericidal or bacteriostatic depending on the nisin concentration, bacterial concentration, physiological state of bacteria and the prevailing conditions (Delves-Broughton et al., 1996). The action of nisin against bacterial spores in the majority of the cases is sporostatic rather than sporicidal and the spores become more sensitive to nisin the more heat damaged they are and lower the pH (Scotland Taylor, 1981a,b). In that case, application of nisin in heat processed foods is feasible, but sufficient residual nisin must be maintained throughout the shelf life of the product (Delves-Broughton et al., 1996).

1.1.7 NISIN RESISTANCE

The resistance of the bacteria to nisin is related to the ability of nisin to reach the cytoplasmic membrane, bind to it, incorporate into it and form the pore. In Gram positive bacteria the fatty acid and phospholipid composition of the cell membrane and the cell wall are involved.

Nisin resistance of Listeria monocytogenes is related to changes in fatty acid composition of the membrane. Nisin resistant Listeria monocytogenes contain significantly more phosphatidylethanolamine and less phosphatidylglycerol and cardiolipin (Ming and Daeschel 1995; Crandall and Montville 1998) and has a lower C15/C17 fatty acid ratio (Mazzotta and Montville 1997). Resistant mutants of Listeria monocytogenes were found to have fatty acids with higher transition temperature, higher percentage of straight-chain, lower percentage of branched-chain fatty acids (Ming and Daeschel, 1993), higher percentage of long-chain and lower percentage of short chain fatty acids (Mazzotta and Montville 1997). All these fatty acid and phospholipid differences are directly related with the fluidity of the cytoplasmic membrane. Less fluid membrane increases the rigidity and might prevent nisin from inserting into the membrane (Crandall and Montville 1998).
The cell envelope plays a significant role in nisin resistance. Davies et al., (1996) found that without a cell wall, the acquired nisin resistance of the *Listeria monocytogenes* resistant mutant was lost. The cell surface hydrophobicity of the resistant strain is lower than the sensitive strain (Ming and Daeschel, 1995; Davies et al., 1996). The different ability of nisin to bind on the cell surface possibly affects the sensitivity. Bhunia et al. (1991) reported that the sensitivity of Gram-positives to bacteriocins compared to Gram-negatives was due to their greater binding to the Gram-positive cell surface. Bacteriocin molecules attach to specific receptors on the cell surface of the Gram positive bacteria, cause conformational alteration of the cell wall in sensitive bacteria, traverse the cell wall, reach the cytoplasmic membrane and finally inactivate the cell (Bhunia et al. 1991; Ray 1993). Davies & Adams (1994) and Ming & Daeschel, (1995) found that nisin-resistant cells bind less nisin than the sensitive ones.

Intact cells of Gram-negative bacteria are generally resistant to the action of nisin. In Gram-negative bacteria the outer membrane is an effective barrier to the penetration of nisin and other large amphiphilic or hydrophobic antimicrobials (Vaara 1992; Ray 1993). Lipopolysaccharide defective mutants of *Salmonella typhimurium* were sensitive to nisin (Stevens et al., 1992b). Gram negative bacteria can be rendered sensitive to nisin by conversion to spheroplasts (Schved et al., 1994). Kordel and Sahl (1986) showed that *Escherichia coli* exhibited nisin sensitivity when the outer membrane was by-passed by osmotic shock. Chelator permeabilized cells of Gram negative bacteria were sensitive to nisin (Stevens et al., 1991; Delves-Broughton 1993; Cutter and Siragusa 1995). Sublethal injury by heating, freezing and high pressure can also sensitise the Gram negatives to nisin (Kalchayanand et al., 1992; 1994). Hence, disruption of the Gram negative cell wall and alteration of outer membrane permeability make the Gram negatives susceptible to nisin.

Other factors affecting nisin action are not related to the bacteria themselves. These concern food factors such as pH, lipid content, presence of di- and trivalent cations and temperature. Nisin is not stable and loses its activity at high pH as was discussed earlier (section 1.1.3). Lipids, and especially phospholipids bind nisin and therefore it is not
available for action (Daeschel, 1990; Jung et al. 1992). Di- and trivalent cations bind to anionic phospholipids resulting in an enhanced rigidity of the cytoplasmic membrane reducing the affinity to nisin (Abee et al., 1994; Crandall and Montville 1998). Low temperatures reduce nisin effectiveness, since ordering of the lipid chains, which occurs at low temperatures, results in a decrease in membrane fluidity and likely affect the antimicrobial ability of nisin (Abee et al., 1994).

1.1.8 APPLICATIONS OF NISIN

The suitability of nisin as a food preservative arises from the fact that is non-toxic (nisin has been ingested naturally in raw milk over the past centuries without ill effect); the producer strains of Lactococcus lactis are regarded as safe (food-grade); it is not used clinically (there is no apparent cross-resistance in bacteria that might affect antibiotic therapeutics); it is digested immediately and it is heat stable at low pH (Delves-Broughton, 1990).

Nisin has become established an effective preservative in pasteurised processed cheese and pasteurized processed cheese spreads. This is because heat processing (85-105°C for 6-10 min) of the raw cheese during melting, is not adequate to eliminate clostridial spores (Clostridium butyricum, Cl. tyrobutyricum, Cl. sporogenes, and Cl. botulinum) present. Generally, levels of nisin used to control non-botulinal spoilage in processed cheese vary from 250 to 500 IU/g and more than 500 IU/g to provide protection against Clostridium botulinum (Delves-Broughton et al., 1996).

In countries with high ambient temperatures and inadequate refrigeration and transport facilities, nisin at levels of 25-50 IU/ml provide a significant increase of shelf life of pasteurised milk. Effective preservation of pasteurised flavoured milks and dairy desserts has also been reported using levels of 100-200 IU/ml (Delves-Broughton, 1990).

Nisin is used in canned foods principally for the control of thermophilic spoilage from Bacillus stearothermophilus and Clostridium thermosaccarolyticum heat resistant
spores. Addition levels of nisin used in canned foods are generally between 100 to 200 IU/ml and product examples include canned vegetables, soups and cereal pudding (Delves-Broughton, 1990).

Nisin is successfully used for the prevention of food poisoning caused by Bacillus cereus in crumpets, where addition of nisin at levels of 15 IU/ml prevents Bacillus cereus from growing to levels capable of causing food poisoning (Jenson et al., 1994).

Other potential applications of nisin include the partial replacement of nitrites in cured meats. Results indicated that only high and uneconomical levels of nisin achieved good control of Clostridium botulinum. Reasons proposed for the poor preservative effect include the binding on to meat particles, uneven distribution, poor solubility and possible interference in the mode of action from phospholipids (Delves-Broughton et al., 1996).

Nisin has also the potential in controlling spoilage lactic acid bacteria in beer, wine and salad dressings but the yeasts are completely unaffected (Delves-Broughton et al., 1996). Addition of nisin into liquid egg products following pasteurisation has shown to act quite effectively and increase the self-life of the product (Delves-Broughton et al., 1992).

Other potential applications could be in combination with other bacteriocins, to increase the antibacterial activity (Hanlin et al., 1993), and with lysozyme and/or chelators (ter Steeg 1993; Shefet et al., 1995) in order to increase the effect and the antimicrobial spectrum of nisin. The use of nisin-producing starter cultures for in situ incorporation of nisin into foods (Harris et al., 1992b; McMullen and Stiles 1996) is also of a great interest. Novel applications of nisin concern incorporation into biodegradable packaging films (Padgett et al., 1998) and the use of nisin as adjunct for non thermal preservation techniques such as high pressure and pulsed electric field (Kalchayanand et al., 1994).
1.2 OUTER MEMBRANE OF GRAM NEGATIVE BACTERIA

The outer membrane is the unique outer part of the cell wall of Gram-negative bacteria. It contains characteristic compounds, e.g. lipopolysaccharides, and serves as a physical and functional barrier. It is involved in aspects of bacterial interaction with the environment, pathogenicity and interaction with the host cell, bacteriophages and acts as a permeability barrier (Nikaido and Vaara 1985). This last property deserves the attention of food microbiologists since Gram-negatives are resistant to a variety of hydrophobic antibacterial compounds which can be food preservatives, natural antimicrobials and bacteriocins (Vaara 1992; Ray 1993).

1.2.1 BACTERIAL CELL WALL

The cell envelopes of prokaryotic microorganisms are characterised by the presence of two distinct components. An inner cytoplasmic membrane, which controls the substrate and electron transport processes of the cell and which is the site of biosynthesis of extracellular macromolecules, and a strong outer cell wall, which maintains the shape of the cell and protects the mechanically fragile cytoplasmic membrane from rupture owing to the high osmotic pressure exerted on it by the cell cytoplasm (Hancock and Poxton, 1988).

Bacteria can be divided into two main classes based upon their reaction to Gram staining. The differences between Gram positive and Gram negative bacteria relate to differences in their cell wall structure and chemical composition. The cell wall of Gram negatives is more complex because they have an additional layer called the outer membrane (OM) (Hammond et al. 1984).

In Gram positive bacteria the cell wall consists of peptidoglycan and secondary wall polymers like, teichoic acids, polysaccharides and proteins that are covalently linked to the peptidoglycan throughout its thickness and loosely associated lipocarbohydrates. (Figure 1.2.1), (Hancock and Poxton, 1988).
In Gram negative bacteria the inner region of the wall is a thin layer of peptidoglycan. The outer region of the wall in some respects resembles the cytoplasmic membrane and is therefore commonly called the outer membrane. The region between the inner and outer wall layers is termed the periplasm and found only in Gram negative bacteria (Figure 1.2.2), (Hancock and Poxton, 1988).

1.2.2 PEPTIDOGLYCAN

Peptidoglycan, which contributes to the mechanical stability of cell walls, is a polymer consisting of a disaccharide-repeating unit of two different N-acetylated amino sugars, to one of which is attached a short peptide chain. Individual glycan strands are cross-linked through peptide bonds between the peptide chains to create a covalent network with great mechanical strength. Figure 1.2.3 shows the structure of peptidoglycan (Hammond et al. 1984).

The peptidoglycan layer is not covalently attached to the underlying cytoplasmic membrane, but in Gram negative bacteria it is firmly bound to the outer membrane by covalent linkage of occasional side-chains to an abundant outer membrane protein called murein lipoprotein (Hammond et al. 1984).

1.2.3. OUTER MEMBRANE COMPONENTS

The outer membrane (OM) of Gram-negatives consists of phospholipids, lipopolysaccharides and proteins like murein lipoprotein, classical trimeric porins, monomeric porins, proteins involved in specific diffusion, high-affinity proteins, and few enzymes.
Figure 1.2.1. The cell envelope of a Gram-positive bacterium. P-protein, PL-phospholipid, 2° wall polymer-techoic acid. [From Hancock and Poxton (1988)]

Figure 1.2.2. The cell envelope of a Gram-negative bacterium. LP-lipoprotein, P-protein, LPS-lipopolysaccharide, PL-phospholipid. [From Hancock and Poxton (1988)]
Figure 1.2.3. Generalized presentation of the structure of bacterial peptidoglycan.

[From Hammond et al., (1984)]
1.2.3.1. Phospholipids

Phospholipids composition of the outer membrane is usually very similar to that of the cytoplasmic membrane but a significant enrichment in phosphatidylethanolamine has been found in *Salmonella typhimurium* (Nikaido and Vaara 1985).

1.2.3.2 Lipopolysaccharides

The lipopolysaccharides (LPS) are the unique constituent of the bacterial OM. They are composed of three parts: (i) the proximal hydrophobic lipid A region, (ii) the distal hydrophilic O antigen polysaccharide region that protrudes into the medium and (iii) the core polysaccharide region that connects the two (Figure 1.2.4). It is also known as endotoxin, since injection of pure LPS or LPS-containing bacteria into experimental animals causes a variety of so called endotoxic reactions. (Luderitz et al., 1983; Nikaido and Vaara 1985).

**Lipid A**

Lipid A consists of a glycosamine disaccharide backbone which links the polysaccharide chain to C-6 of a non-reducing glucosamine residue, and six or seven fatty acid chains linked to the disaccharide. All of the fatty acid chains are saturated and some are 3-hydroxy fatty acids. Furthermore, some fatty acid residues are linked to the 3-hydroxy group of another fatty acid, producing the characteristic 3-acyl-oxy-acyl structure (Figure 1.2.5), (Takayama et al., 1983). *Salmonella* lipid A contains 7 molecules of long-chain fatty acids. Four molecules of D-3-hydroxytetradecanoic acid (β-hydroxy-myristic acid) and one of dodecanoic (lauric), tetradecanoic (myristic) and hexadecanoic acid (palmitic acid) (Luderitz et al., 1983). D-3-hydroxytetradecanoic acid exists not only in the lipid A of the Enterobacteriaceae family but also in most of the Gram-negative bacteria. The lipid A of pseudomonads lacks this characteristic constituent. The major hydroxy fatty acid is the D-3-hydroxydodecanoic acid (β-hydroxylauric acid), (Meadow, 1975). Many negatively charged groups exist on the backbone itself, as well as on proximal sugar residues, such as phosphate groups and deoxy-D-manno-octulosonic acid (KDO). Indeed, LPS was found to bind divalent...
Figure 1.2.4. Generalized structure of bacterial lipopolysaccharide. [From Hammond et al., (1984)].

Figure 1.2.5. Structure of Lipid A from the LPS of Salmonella typhimurium. The dotted line shows the attachment of C-6 of the non-reducing glucosamine residue to the polysaccharide chain. [From Takayama et al., (1983)].
cations quite strongly, and the nature of counter ions has a profound influence on the physical structure of LPS aggregates (Nikaido and Vaara, 1985).

Core polysaccharide
The core polysaccharide, like lipid A, is quite uniform. The core of Salmonella typhimurium, for instance, is common to all Salmonella species. The Salmonella core contains a lipid A-distal hexose oligosaccharide consisting of D-glucosamine, D-glucose, and D-galactose, and the inner lipid A-proximal region consisting of an oligosaccharide of the core specific sugars, L-glycero-D-manno-heptose (L-D-Hep) and the unique negatively charged 3-deoxy-D-manno-octulosonic acid (KDO), each forming a branched trisaccharide (Luderitz et al., 1983).

O-antigen polysaccharide
The O-specific chains of lipopolysaccharide are made up of repeating units of identical oligosaccharides. These units usually contain different constituents, thus the O chain represents a heteropolysaccharide. In some cases, however, the repeating unit may contain an oligomer of a single sugar type though in a distinct linkage sequence, hence repeating units can also be recognised. In these cases the O chain represents a homopolysaccharide. The O chains contain the immuno-determinant structure against which the anti-O antibodies formed during infection or on immunization are directed. Each bacterial serotype synthesizes a unique LPS. Characterized by a specific composition and structure of the O chain and by an individual O antigenicity (Luderitz et al., 1983).

1.2.3.3 Proteins

Nearly half of the mass of the OM is protein. Most membrane proteins are located in the OM, but some of them are found in both the outer and cytoplasmic membranes (Nikaido and Vaara, 1985). A few ‘major’ proteins usually dominate the protein profile. These include the porins and the murein lipoprotein.
Murein lipoprotein

Murein lipoprotein is a small (7,200-dalton) protein that exists in a large number of copies, 7x10^3 per cell (Nikaido and Vaara 1985). A great proportion of these (approximately one third) are bound covalently to the peptidoglycan layer through the -NH_2- group of its C-terminal lysine (Inouye et al., 1972), and the -NH_2- group of its N-terminal cysteine is substituted with a fatty acid through an amide linkage (Braun 1975). The polypeptide chain appears to exist mostly as α-helical form (Braun et al., 1976). The lipoproteins tend to form cross-links with each other (Reithmeyer and Bragg, 1977). The lipoprotein is not essential for growth since lipoprotein-negative mutants are quite viable (Hirota et al. 1977) and the diffusion of small hydrophilic solutes is normal (Nikaido et al., 1977), but the cell wall structure in these mutants appears to be unstable, resulting in the release of outer membrane vesicles and periplasmic enzymes (Hirota et al. 1977). Hence, the main function of the protein is structural stabilizing the architecture of the outer membrane-peptidoglycan complex by holding down the outer membrane to the surface of the peptidoglycan (Nikaido and Vaara 1985).

Classical Porins

The proteins coded for by OmpC, OmpF and PhoE genes in E.coli K-12 and OmpD in Salmonella typhimurium are called porins because they produce relatively non-specific pores or channels that allow the passage of small hydrophilic molecules across the outer membrane (Nikaido 1996). Porins forms large (0.6-2.3 nm diameter) trans-outer membrane, water-filled channels (Hancock 1987) of which the physiological importance in allowing diffusion of nutrients, antibiotics or inhibitors across an outer membrane has been established by the use of porin-deficient mutants (Lutkenhaus 1977). The porins allow the influx of nutrients and efflux of hydrophilic waste products smaller than 600 daltons (Nakae and Nikaido 1975). Porins have monomer molecular weights in the range of 28-48 kDaltons and exist usually as trimers (Nakae et al., 1979), rich in β-sheet structure. In OmpC and F the trimers form 3 openings at the surface of the outer membrane. However the three separate openings merge into a single channel near the mid-point of the membrane (Engel et al., 1985). In contrast, in PhoE, each monomer forms transmembrane channels in their own right. Figure 1.2.6 shows the schematic representation of the porins. Crystal structures of porins show that they consist of trimers.
of β-barrels consisting of antiparallel β-strands that pass through the outer membrane (Hancock 1997). More specifically, the polypeptide chain of its subunit traverses the membrane 16 times as antiparallel β-strands, forming a β-barrels structure surrounding a large channel. Each subunit produces a channel and the trimer therefore contains three channels (Cowan et al., 1992) (Figure 1.2.7)

Monomeric porins (OmpA and OprF protein)
In contrast of the classical trimeric porins, the OmpA protein of E.coli and OprF of Pseudomonas aeruginosa are examples of monomeric porins, which also show slower diffusion rates than trimeric porins (Nikaido 1993). Monomeric porins are almost as abundant as porins, hence, each cell contains about 10^5 copies. OmpA protein of E.coli, has a monomer molecular weight similar to that of porins, but behaves very differently upon solubilization in sodium dodecyl sulfate and, like the porins, is rich in β-sheet structure, and appears to span the thickness of the membrane (Nikaido and Vaara 1985). OmpA mutants lacking the OmpA protein are extremely poor recipients in conjugation and produce an unstable outer membrane (Nikaido and Vaara 1985). The penetration of solutes through the monomeric OmpA channel is about two orders of magnitude slower than through an OmpF channel and thus OmpA does not make a significant contribution to permeability (Nikaido 1996). In Pseudomonas aeruginosa the main porin is the OprF which is also monomeric and with low permeability like OmpA. This explains the low permeability of Ps. aeruginosa outer membrane (Nikaido 1996).

Specific transport proteins and High-Affinity receptors
Many of the less abundant proteins of the outer membrane are specific transport proteins or receptors that mediate the entry of molecules unable to pass through the regular porins (Nikaido and Vaara 1985). The LamB protein of E.coli is involved in allowing passage of maltose and maltodextrins through the outer membrane and is a porin-like trimeric protein constructed as a β-barrel (Nikaido 1996). The structural similarity suggests that both channels operate by a similar mechanism but there is a specific binding site for maltose inside LamB channel (Nikaido 1993). Other specific channels in E.coli are the Tsx and ScrY proteins, which form specific channels for nucleosides and sucrose respectively (Nikaido 1996). High-affinity receptor proteins catalyse specific transport of
Figure 1.2.6. Schematic representation of the structures of different porin proteins. [From Hancock (1987)].

Figure 1.2.7. Folding pattern of OmpF porin. (A) A diagram showing a monomeric unit, which consists of 16 transmembrane, antiparallel β-strands. (B) View of the OmpF trimer. [From Nikaido (1996)].
ligands across the OM. These proteins are differentiated from the specific transport proteins by binding the ligands much more tightly and hence are called 'receptors'. The transport of ligands through these proteins requires the presence of TonB protein, which is anchored in the cytoplasmic membrane (Nikaido 1996). Examples are the BtuB protein, which binds vitamin B₁₂ and FadL protein which is responsible for the diffusion of long-chain fatty acids across the OM (Nikaido 1996).

Other proteins
A few enzymes are located in the outer membrane. They include phospholipase A₁ and proteases (Nikaido and Vaara 1985).

1.2.4 OUTER MEMBRANE STRUCTURE AND FUNCTIONS

The OM has an asymmetric bilayer structure where only the inner leaflet contains phospholipid and the outer leaflet LPS molecules, which are held close together with the aid of divalent cations, forming a 'tilted roof' (Vaara 1992).

The outer leaflet of the bilayer contains almost exclusively LPS (Funahara and Nikaido 1980; Mulford and Osborn 1983) and is virtually free of phospholipids (Kamio and Nikaido 1976). Schindler and Osborn (1979), showed that the anionic groups in the LPS are arranged in such a way that they accommodate divalent cations. Removal of the cations by electrodialysis and reintroduction of known cations, showed the decisive influence the cations have on LPS stability (Galanos and Luderitz, 1977). Takeuchi and Nikaido (1981) showed that the LPS-LPS interaction is very strong in the presence of Mg^{2+}. Chelation of the divalent cations by the action of EDTA liberated almost 50% of the LPS of E. coli and made the cells sensitive to hydrophobic antibiotics (Leive 1974).

The lipid A of the LPS has also a highly ordered conformation compared to phospholipids and this high state of order is important to the structural role and
permeability barrier functions of the OM (Labischinski et al., 1985). Indeed, the large number of hydrocarbon chains (each Lipid A molecule contain six to seven saturated fatty acids), increases the contact, decreases the intermolecular mobility and results in low fluidity, which enhances the structure and the low permeability properties of OM (Nikaido 1996). In contrast the negatively charged groups of the LPS, which are located on the lipid A and inner core polysaccharide region, induce repulsion to the neighbouring LPS, but the repulsion is neutralised by the divalent cations (Nikaido 1996).

Hence, the molecular basis of the OM integrity is based mainly on the binding of divalent cations (Ca $^{2+}$ and Mg $^{2+}$) by the polyanionic LPS. Nevertheless, excessive levels of divalent cations, especially Ca $^{2+}$ disrupt the OM, probably by ‘freezing’ the LPS, where increasing their thermal transition temperature, produces ‘cracks’ in The OM (Nikaido and Vaara 1985). Apart from the interactions between LPS-LPS, which stabilise the OM, interaction between LPS and OM proteins occurs and contribute to the OM stability. This is apparent from the fact that mutants lacking the OmpA protein produce unstable outer membrane and ‘deep rough mutants’, which are hypersensitive to various compounds, contain reduced amounts of OM proteins (Nikaido and Vaara 1985).

There are mutants that produce defective LPS molecules (rough mutants), (Figure 1.2.8). Mutants with Re and Rd chemotypes are called ‘deep rough’ mutants and contain large amounts of phospholipids on their outer leaflet. The level of the OM proteins is reduced in deep rough mutants (Ames et al., 1974; Smit et al., 1975). The phospholipid content is increased and the LPS content is constant, regardless the length of saccharide chain (Smit et al., 1975) or the LPS content is little increased (20-30%) especially in the stationary phase cells, probably due to blebbing or protrusions of the OM (Nikaido and Vaara 1985). These mutants, according to the model of Nikaido and Vaara (1985), are sensitive to hydrophobic compounds. These compounds can now pass mainly through the phospholipid region, or is assumed to penetrate the OM through the LPS domains, which have become more permeable due to the looser LPS-LPS and LPS-OM proteins interaction of the ‘deep rough’ mutants (Figure 1.2.9.). These mutants can become less
Figure 1.2.8. Structure of LPS from *Salmonella typhimurium* LT2 and its mutants. Ra through Re refer to the chemotypes of the mutant LPS produced.

[From Nikaido (1996)].
Figure 1.2.9. Hypothetical structure of OM in the wild type, deep rough mutants and EDTA-treated wild type cells of Enterobacteriaceae. (A) Wild-type strain in which the OM is almost entirely composed of LPS and proteins. (B) and (C) Deep rough mutants. Hydrophobic molecules are assumed to penetrate through the phospholipid bilayer domains (B) or through the LPS domains, which could have become more permeable due to the alterations in LPS structure (C). (D) EDTA-treated wild-type cells. Phospholipid molecules are assumed to fill the void space left by the removal of LPS by EDTA. [From Nikaido and Vaara (1985)].
sensitive to hydrophobic compounds by addition of cations into the growth medium
(Stan-Lotter et al., 1979).

The integrity of the asymmetric bilayer confers a permeability barrier to a variety of
molecules, especially hydrophobic or amphiphilic molecules, since small hydrophilic
compounds are able to pass through porins (Nikaido 1976). The passage rate through
the molecular sieve of porins is dependent on solute size, electric charge, hydrophobicity
and specific receptors that exist for specific solutes (Nikaido and Vaara 1985). Large,
hydrophobic or amphiphilic compounds have to pass through the asymmetric bilayer of
OM. Although the OM is generally an effective barrier to amphiphilic compounds, it is
not 100% impermeable to uncharged hydrophobic compounds (Nikaido 1996). Earlier
studies have shown that the OM allows slow influx, with permeability rates about 50-100
times less than the cytoplasmic membrane permeability (Plesiat and Nikaido 1992). The
resistance of Gram-negatives to such compounds is also due to specific efflux systems
(Hancock 1997; Nikaido 1998). These efflux systems are believed to consist of an
energized low-specificity cytoplasmic pump, a periplasmic ‘link’ protein and an outer
membrane ‘pore’ protein (Figure 1.2.10). (Hancock, 1997). The efflux systems, together
with periplasmic enzymes like β-lactamases, are also responsible for the resistance of
Gram-negatives to antibiotics like β-lactams antibiotics (Hancock 1997; Nikaido 1998).

Summarising, the permeation of compounds into the Gram-negative bacteria occurs for
small hydrophilic compounds through porins, for large hydrophobic compounds through
LPS, with slow rates in wild-type cells and faster rates in ‘deep rough’ mutants (due to
the phospholipid patches and/or reduced LPS-LPS interaction), and by ‘self-promoted
uptake’ in the case of cationic peptides. The last issue is discussed in detail in the
following section. Their relative resistance is conferred by the LPS asymmetric bilayer,
which excludes large amphiphilic compounds and slows down the uptake of uncharged
hydrophobic compounds, the efflux pumps and the presence of periplasmic enzymes like
β-lactamases.
Figure 1.2.10. Schematic diagram of the cell envelope showing the uptake and resistance mechanisms. (1) porin pathway for the small hydrophobic molecules. (2) slow influx for uncharged hydrophobic compounds and self promoted pathway for polycations. (3) Three-component antibiotic efflux system. (4) periplasmic β-lactamase.
1.2.5 OUTER MEMBRANE PERMEABILITY ALTERATIONS

The outer membrane permeability barrier is prone to alterations under various conditions. Excluding mutations, chemical treatment with compounds which can act as permeabilizers (chelators, cationic peptides etc) or physical treatment like heating, freezing-thawing, freeze-drying, osmotic shock etc, can damage the outer membrane barrier and alter outer membrane permeability (Hancock 1984; Vaara 1992).

1.2.5.1 Effect of chelators and cationic peptides

Chelators, especially EDTA, have a profound effect on the OM permeability barrier of Gram negative enteric bacteria and pseudomonads. They remove, by chelation, the stabilizing divalent cations from their binding sites in LPS. This results in the release of a significant proportion of LPS (40-50%) from the cells (Leive, 1974). The effect of EDTA and other chelators on sensitisation of Gram-negatives to hydrophobic compounds can be explained according to the previously mentioned model of Nikaido and Vaara (1985). EDTA chelates cations and releases LPS. Hence, the permeability increases because the space formerly occupied by LPS, is filled by phospholipids, or by the weakening of the LPS-LPS interactions. Other compounds that act as chelators are hydroxylmethyl-nitromethane (Tris) and sodium hexametaphosphate (HMP) (Vaara 1992).

Various cationic peptides can act as OM permeabilizers. These include polymyxin antibiotics, aminoglycoside antibiotics, lysine polymers, protamine and transferrins (Hancock 1984; Vaara 1992). Polymyxins are pentacationic amphipathic lipopeptide antibiotics, characterized by a heptapeptide ring and a fatty acid tail. The polymyxins are bactericidal even against healthy Gram negative bacteria by a dual mechanism of action (Vaara 1992). They bind to the OM, and interact with the divalent cation binding sites and because they have higher affinity for these sites than the cations, displace them and permeabilize the OM (sublethal action) by ‘self promoted’ uptake (Hancock 1997). This allows it to enter the cytoplasmic membrane where it causes leakage of cytoplasmic components (lethal action) (Vaara 1992). Polymyxin derivatives, which lack the fatty
acid tail (Polymyxin nonapeptide), are less or not bactericidal at all, but preserve the OM-permeabilizing action and sensitise the Gram-negatives to hydrophobic antibiotics. (Vaara and Vaara, 1983a). Polymyxins release little LPS compared to EDTA (Vaara and Vaara 1983b). Protamine and lysine were also found to release 20-30% of the LPS from a Salmonella typhimurium smooth strain and sensitise the bacteria to SDS (Vaara and Vaara 1983b). The same effect is caused by aminoglycosides, which interact with divalent-cation binding sites on Pseudomonas aeruginosa LPS and permeabilize the outer membrane (Hancock 1984). Lactoferrin and transferrin damage the OM of Gram-negative, release LPS and the damage is modulated by Ca\(^{2+}\) and Mg\(^{2+}\) (Ellison et al., 1988; 1990).

The majority of the evidences suggest that the permeabilizing properties of chelators and cationic peptides is related to interaction with divalent cations binding sites of LPS. Hydrophobic antibiotics, EDTA and polymyxin resistant mutants of Salmonella typhimurium lack phosphate groups in the core oligosaccharide (Helander et al., 1997) or have their ester-linked phosphate groups in the lipid A substituted with 4-amino-4-deoxy-L-arabinose in a degree of 60-70% compared to 10-15% in the corresponding wild type lipid A (Vaara 1981; Vaara et al., 1981). The mutation eliminates negative charges and LPS-LPS interactions mediated by divalent cations. This, both reduces binding of cationic polymyxins on anionic LPS and EDTA chelating and permeabilizing activity (Vaara 1981).

### 1.2.5.2 Effect of physical treatments

Injury by physical treatments renders Gram-negative bacteria sensitive to a variety of compounds (bile, lysozyme, hydrophobic antibacterials and antibiotics), implying damage of the OM permeability barrier. The LPS seems to be a key molecule involved in OM damage. Heating induced release of lipopolysaccharides and other OM materials (Hitchener and Egan 1977; Katsui et al., 1982; Tsuchido et al., 1985), and freezing caused conformational alterations and structural damage to LPS of the OM (Kempler and Ray 1978). Damage of the permeability barrier and outer membrane of Gram negatives by physical treatments is discussed more extensively in the following section.
1.3 BACTERIAL INJURY

1.3.1 DETERMINATION OF INJURY

Exposure of bacteria to an adverse environment (e.g. physical treatments or chemical factors) results in a decline in their population (Postgate 1976). Straka and Stokes, (1959), observed that bacterial populations exposed to freeze-thawing stress, contain not only dead and normal cells but also an intermediate state, which was defined by the different nutritional requirements for growth. Straka and Stokes (1959) found that after the treatment, there were cells able to grow on complex agar medium, rich in peptides, amino-acids, vitamins, purines and pyrimidines, but unable to grow on minimal medium composed of inorganic salts and glucose. They defined the cells that were unable to grow on minimal agar as ‘metabolically injured’ due to their impaired ability to synthesise essential cellular components. In the studies of Ray et al., (1971) and Ray et al., (1972), it was found that freeze-drying and freeze-thawing injured cells of Salmonella anatum respectively, were unable to grow on agar medium containing deoxycholate and were sensitive to lysozyme, implying structural damage, especially of the cell wall. These cells, which were sensitive to surface active compounds of selective media, were designated as ‘structurally injured’ (Ray and Speck 1973).

Hence, damage to the permeability barrier renders bacterial cells sensitive to many selective compounds and some in addition, have damage to functional components that are related to their metabolic activities. As long as the extent of the damage is not too great, the injury is reversible (sublethal injury), when it goes beyond a certain level the injury is non-reversible (lethal injury) (Ray 1979). According to Ray and Speck (1973) a stressed population contains:

* Lethally injured (dead) cells: those, which fail to grow on a non-selective complete medium.
* Uninjured cells: those, which grow well on any agar, (complete, minimal or selective).
* Sublethally injured cells: those, which grow on complete non-selective agar only.
The last two groups comprise the survivors. The injured cells can be distinguished to metabolically and structurally injured according to the media used for their detection (selective or minimal). Hence they can be characterised as:

- **Structurally injured**: those that are unable to form colonies on selective agar media.
- **Metabolically injured**: those that are unable to form colonies on minimal agar media.

These terms are not 100% acceptable, since there have been reported cases, where the cells recovered better in 'simple and poor' rather than in 'complex and rich' media (Gomez *et al.*, 1973; Hurst *et al.*, 1973; Departmental research, unpublished data).

### 1.3.2 REPAIR

The ability of injured cells to form colonies on complex media as compared to minimal is due to the recovery and repair that takes place in the presence of suitable compounds. Straka and Stokes (1959) showed that the injured cells are able to recover and repair when supplemented with various peptides. Ray *et al.*, (1972) found that despite the repair of few cells in some minimal media, substantial recovery took place in a complex broth. Complex medium was required for the recovery and repair of *Staphylococcus aureus* and *Salmonella typhimurium* injured by heat treatment (Iandolo and Ordal 1965; Clark and Ordal 1969). Generally, most injured cells repair within 2 h at a suitable incubation temperature in a nutritionally rich, non-selective medium and the repaired cells regain the characteristics of normal cells, including the ability to multiply (Ray, 1986). Adenosine triphosphate (ATP) synthesis is required for repair (Ray *et al.*, 1971; Ray and Speck 1972). However, there have been reported cases where the microorganisms recovered more readily in minimal than complex media (Gomez *et al.*, 1973; Hurst *et al.*, 1973).
1.3.3 SITES OF BACTERIAL INJURY

Various sites are prone to damage after injury by physical treatments. Irrespective of the differences in sublethal treatments and bacterial species, there are similarities in bacterial cell damage and repair as can be seen below.

<table>
<thead>
<tr>
<th>Manifestation and sites of injury</th>
<th>freezing</th>
<th>heating</th>
<th>drying</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss of cellular material</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sensitivity to selective agents</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Wall components</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cell membrane</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DNA, RNA</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Recovery and Repair</th>
<th>freezing</th>
<th>heating</th>
<th>drying</th>
</tr>
</thead>
<tbody>
<tr>
<td>De novo synthesis of:</td>
<td></td>
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<tr>
<td>Peptidoglycan</td>
<td>-</td>
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<tr>
<td>RNA</td>
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<tr>
<td>Protein</td>
<td>-</td>
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</tr>
<tr>
<td>ATP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Reorganisation of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS (Gram-negatives)</td>
<td>+</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>Teichoic acids (Gram-positives)</td>
<td>+</td>
<td>+</td>
<td>?</td>
</tr>
</tbody>
</table>

(Adapted from Ray 1986)

Damage to membranes is very common in treatments related to heating, freezing-thawing, cold shock and freeze-drying. Loss of absorbing material at 260-280 nm has been recorded in thermal-injured (Iandolo and Ordal, 1966; Russel and Harries 1967),
freezing-damaged (Calcott and MacLeod, 1975) and chilling-stressed bacteria (Strange and Ness, 1963). Freeze-dried cells of *Lactobacillus acidophilus* also exhibited cell membrane damage (Brennan et al., 1986).

Damage to DNA (single strand breaks) and to ribosomes by heat, has been shown by Bridges et al., (1969) and by Weiss and Tal (1973) respectively. Damage to biochemical function has also been reported. Not only heating, which is known to denature proteins and enzymes, but also low temperature stress affects enzymes by destabilising the weak bonds between protein molecules (Cowman and Speck 1969).

Cell wall and permeability damage is apparent in physical stresses, since both Gram-positive and Gram-negatives become sensitive to compounds that they are usually resistant to. Heat injured *Staphylococcus aureus* was found to be able to repair in the presence of penicillin but not in actinomycin D (Iandolo and Ordal, 1966). Cold shock and heat treatment increase the sensitivity of *Clostridium perfringens* to neomycin and various selective agents (Traci and Duncan 1974; Barach et al., 1974). *Lactobacillus acidophilus* became sensitive to bile and lysozyme after freezing and freeze-drying respectively (Johnson et al., 1984; Brennan et al., 1986). In a study of thermal injury of *Staphylococcus aureus* it was found that magnesium was lost from the cell wall during heating. Cells containing higher levels of wall teichoic acids, showed less injury than normal cells and cells with weaker cation-binding polymer such as teichuronic acid showed greater injury, attributing the effect of magnesium in stabilising the cell wall against injury (Hoover and Gray 1977). Johnson et al., (1984), found that the freeze injury of the cell wall of *Lactobacillus acidophilus* was reversible and associated with repair of cell wall components other than peptidoglycan.

The permeability barrier and the outer membrane of Gram-negatives become damaged because of physical treatments. Gram-negative bacteria injured by heating, freezing, and freeze-drying become sensitive to bile and deoxycholate (Clark and Ordal 1969; Ray et al., 1971; Ray et al., 1972). Chilling rendered *Aerobacter aerogenes* permeable to a hydrophobic fluorescent dye (Strange and Postgate 1964). Heating induced leakage of
periplasmic enzymes (Katsui et al., 1982; Tsuchido et al., 1985) sensitivity to hydrophobic antibacterials (Tsuchido and Takano 1988), and release of lipopolysaccharides and other OM materials (Hitchener and Egan 1977, Katsui et al., 1982; Tsuchido et al., 1985). Freezing caused release of periplasmic enzymes (Calcott and MacLeod 1975), sensitivity to hydrophobic and surface active compounds (Ray et al., 1976) and conformational alterations and structural damage of LPS of the OM (Kempler and Ray 1978).

Summarising, injury by physical treatments renders Gram-negative bacteria sensitive to a variety of compounds (biles, lysozyme, hydrophobic antibacterials and antibiotics) that they are usually resistant to, implying damage of the OM permeability. The LPS seem to be a key molecule related to OM damage. Damage of outer surface by changing their conformation and/or releasing LPS molecules seems to be common attribute among cells injured by heating and freezing, since properties like cell surface hydrophobicity and adsorption of phages are altered (Ray et al., 1976; Mackey 1983; Tsuchido et al., 1985). Furthermore, ‘deep rough’ mutants of Salmonella typhimurium, injured by freeze-thawing are more sensitive to SDS, EDTA and various hydrophobic antibiotics than ‘wild type’ strains (Bennett et al., 1981), confirming the significance of LPS in OM barrier integrity.
1.4 AIM OF THE PROJECT

Nisin is a well-established natural food preservative approved by the food legislation in many countries and used in a variety of foods. The target organisms though, are important Gram-positive bacteria, vegetative and spores. Gram-negative bacteria are generally resistant to hydrophobic and amphiphilic compounds like nisin, because of the existence of OM permeability barrier that they posses. However, Gram-negatives become sensitive to such compounds when their OM is injured and its permeability barrier has been overcome. It is important from the technological point of view to study the expansion of use of nisin against those microorganisms.

This project investigates the ability of nisin to inhibit or/and inactivate Gram-negative bacteria, injured by processes currently used for food preservation. Treatments like fermentation with nisin produced in situ, heating, freezing, and chilling, where nisin can be used as adjunct to these treatments, might be able to reduce the population or inhibit the growth of these bacteria, making the processes more effective. Studies in laboratory media and real food systems are necessary to explore the synergistic effect of nisin and other compounds and processes against Gram-negative bacteria important in food safety and preservation such as Salmonella Enteritidis, Escherichia coli and Pseudomonas species. Furthermore, studies of the effect of these treatments on the outer surface and permeability barrier are essential in order to study the nature of damage that renders these bacteria sensitive to nisin.
CHAPTER 2

GENERAL MATERIALS AND METHODS
CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Organisms

The microorganisms that were used during the project were the following:

*Lactococcus lactis* NCFB 497 (nisin producer), and NCFB 712 (non nisin producer) taken from The University of Surrey collection

*Salmonella* Enteritidis PT4, P167807, supplied by Division of Enteric pathogens, Central Public Health Laboratory, London, UK.

*Salmonella* Enteritidis PT7, P469815, a strain with shorter lipopolysaccharide chain length (Chart *et al.*, 1989), was also supplied by The Division of Enteric pathogens, Central Public Health Laboratory, London, UK.

*Escherichia coli* ATCC 25922, taken from The University of Surrey collection

*Escherichia coli* 001, 089, 102 and 105, taken from our Food laboratory collection

*Pseudomonas aeruginosa* USCC 2186, and ATCC 27853, taken from The University of Surrey collection

*Pseudomonas fragi* ATCC 4973, taken from The University of Surrey collection

*Micrococcus luteus* NCIB 8166, for the nisin bioassay was donated by Aplin and Barrett Ltd, Dorset, UK.

All the bacteria were stored frozen on beads (Protect; Technical Service Consultants Ltd, Heywood, Lancashire, UK) at -70°C. For resuscitation, one bead was added to 10 ml Nutrient Broth or M17 Broth for lactic acid bacteria and incubated at 37°C or 30°C (lactic acid bacteria) or 25°C (*Pseudomonas fragi*) for 24h.
2.2 Microbiological media

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

2.3 Nisin

Nisin was supplied in a purified form (5x10^7 IU/g) by Aplin and Barrett Ltd, Dorset, UK. Solutions of nisin were prepared in 0.02N HCl (pH 2) sterilized by filtration through 0.45 μm filters (Minisart, NML, Sartorious, USA) and stored at 4°C.

2.4 Nisin bioassay

The quantitative agar diffusion assay was used for the estimation of nisin (Tramer and Fowler 1964; Fowler et al., 1975). Nisin standard stock solution was produced by dissolving pure nisin in 0.02N HCl (pH 2) and sterilized by filtration. Accurate weighing and volumetric flasks must be used. Standards of 1.25, 2.5, 5.0, 10, and 20 IU/ml nisin were prepared by accurate dilutions with 0.02N HCl. The test organism, after resuscitation, was grown on slopes of the assay medium and used for the bioassay within 24 h.

The assay medium contained (grams per litre R.O. water): bacteriological peptone 10, Lab-lemco 3, sodium chloride 3, yeast extract 1.5, natural, raw cane sugar 1.0, and Agar No. 1. 10. The pH of the medium was adjusted to 7.5±0.1 prior to sterilization.

The inoculum of test organism (Micrococcus luteus NCIB 8166) was prepared by emulsifying the growth from the slope with 10 ml 1/4 strength Ringer’s solution. The suspension was adjusted to give a transmission of 50% (or Absorbance of 0.3) in a 1.5 ml narrow path cuvette at 650 nm.

The prepared assay medium was melted and cooled to approximately 50°C. Two percent (v/v) of 1:1 mixture of Tween 20 and sterile distilled water, previously held at
50°C for 20 min, was added and thoroughly mixed with the medium. Inoculum of 2% (v/v) was also added to the medium and then placed into the water-bath of 50°C for 10 min to temper.

The medium was poured into an assay plate to a depth of 3-4 mm. After the agar had set, the plate was inverted and stored at 4°C for 1 h to facilitate the cutting of the wells. Wells were punched in the agar using a cork borer of 7-9 mm diameter, allowing 30 mm between adjacent wells and 20 mm between peripheral wells and the edge of the assay plate. Equal volumes (0.1 ml) of standard and test solutions were delivered into the wells and the plate incubated at 30°C for 18-24 h.

The diameter of the inhibition zones was measured by using a digital calliper (CamLab Ltd, Cambridge UK). Calibration graph produced by diameter of zones (mm) against the logarithm of the nisin concentrations was plotted.
CHAPTER 3

EFFECT OF CHELATORS AND NISIN PRODUCED IN SITU

ON INHIBITION AND INACTIVATION

OF GRAM NEGATIVES
CHAPTER 3

EFFECT OF CHELATORS AND NISIN PRODUCED \textit{IN SITU} ON INHIBITION AND INACTIVATION OF GRAM NEGATIVES

The work in this chapter has been published under the title 'Effect of chelators and nisin produced \textit{in situ} on inhibition and inactivation of Gram negatives', in the International Journal of Food Microbiology 53 (1999) 105-113.

3.1 INTRODUCTION

Nisin is a bacteriocin produced by \textit{Lactococcus lactis} subsp. \textit{lactis}. Together with acids and hydrogen peroxide, it is among the biological inhibitors produced by lactococci (Adams 1990). Partially purified nisin is used as a food preservative in a range of products (Delves-Broughton 1990). The potential of nisin produced \textit{in situ} for the preservation of foods, has also been investigated. The target organisms in these cases were important Gram positives like, \textit{Listeria monocytogenes}, \textit{Staphylococcus aureus} and \textit{Clostridium sp}. Wessels and Huss (1996) reported the effect of \textit{in situ} nisin production against \textit{Listeria monocytogenes} in lightly preserved fish products and Stechini \textit{et al} (1995) in Mozzarella cheese. Zottola \textit{et al} (1994) reported that, in cheddar cheese made with nisin-producing strains of \textit{Lactococcus lactis}, the growth of undesirable microorganisms like \textit{Listeria}, \textit{Staphylococcus} and \textit{Clostridium} was better controlled. Although, in some cases the effect has been reported as insignificant (Yusof \textit{et al}., 1993).

Nisin is not generally active against Gram-negative bacteria, yeasts and fungi. The outer membrane (OM) of Gram negative bacteria does not allow molecules like nisin to reach its site of action, the cell membrane. Treatments with chelators can alter the outer membrane permeability of Gram negatives (Hancock 1984; Vaara, 1992). Chelators sequester the Mg\textsuperscript{2+} and Ca\textsuperscript{2+} divalent cations, which stabilise the lipopolysaccharide molecules. In such cases Gram negatives do show sensitivity to nisin. Stevens \textit{et al} (1991; 1992a), Delves-Broughton (1993) and Cutter and Siragusa (1995a) showed that treatments with nisin and chelators reduces the population of Gram negatives.
It has also been reported that treatments of Gram negatives with organic acids like lactic, acetic, malic etc., rendered them sensitive to bile (Roth and Keenan 1971) and storage at 4°C in the presence of acids and nisin reduced their population further than acids alone (Kalchayanand et al., 1992).

Research on sensitisation of Gram negatives to nisin has mainly used chelators in buffers and the addition of exogenous nisin. A few studies have looked at the use of nisin and chelators for decontamination of meat or poultry carcasses (Cutter and Siragusa 1995b; Shefet et al., 1995). The effect of naturally generated nisin against the Gram negatives has not however been studied. The effect of nisin, produced *in situ* by *Lactococcus lactis* against Gram negatives in the presence of chelators and lactic acid is potentially of greater importance, since the major foodborne pathogens are Gram negative.

In this chapter, the effect of nisin produced by *Lactococcus lactis* NCFB 497 and chelators such as EDTA and pyrophosphate in the presence of developed acidity (lactic acid) against Gram negatives in laboratory media was investigated.

### 3.2 MATERIALS AND METHODS

#### 3.2.1 Organisms

For this study, *Escherichia coli* ATCC 25922, *Escherichia coli* 001, 089, 102 and 105, *Salmonella Enteritidis* PT4, P167807, *Pseudomonas aeruginosa* ATCC 27853, *Lactococcus lactis* NCFB 497 and 712, nisin and non nisin producers strains respectively, were used. For the nisin bioassay *Micrococcus luteus* was the target organism. Details about the microorganisms and their maintenance found Chapter 2.

#### 3.2.2 Nisin.

See General Materials and Methods (Chapter 2).
3.2.3 Assay of nisin produced by *Lactococcus lactis*

Nisin produced by *Lactococcus lactis* NCFB 497 was determined by horizontal agar plate diffusion according to Fowler et al. (1975). Fresh inoculum of stationary phase *L. lactis* NCFB 497 from M 17 broth was used to inoculate 10 ml NB No2 containing 1% glucose. After overnight incubation at 30°C, the culture was added at a level of 1% to fresh NB No2 containing 1% glucose to give an initial population of approximately $10^6$ cfu/ml (confirmed by enumeration). The culture was incubated for 12 hours at 30°C. Samples were taken at 0, 4, 8 and 12 hours. They were centrifuged at 1500xg for 15 min, 1 ml of the supernatant diluted as appropriate in 0.02M HCl (pH 2) and 0.1 ml of each sample was transferred to the wells in the assay medium. For the control readings, the samples were adjusted to pH 11 with NaOH, heated for 30 min at 63°C, to inactivate the nisin, cooled down immediately, the pH adjusted at 2 with concentrated HCl, diluted as appropriate in 0.02M HCl and finally 0.1 ml of each sample used for the bioassay. Standard nisin solutions of 1.25, 2.5, 5, 10, and 20 IU/ml were also made in order to correlate the inhibition zone to nisin concentration of the sample by using a calibration curve. The bioassay medium, the test organism used and the details of the methods are described in Chapter 2.

3.2.4 Chelators

The chelators used were disodium EDTA, disodium pyrophosphate, citric acid and sodium hydrogen orthophosphate. Disodium pyrophosphate was supplied by Sigma (Sigma-Aldrich Ltd, Dorset, UK) and the others by Fisons (Fisons, FSA Laboratory Supplies, Loughborough, UK). Concentrated solutions were prepared by dissolving the chelator in R.O. water and filter sterilizing through 0.45 µm filters (Minisart, NML, Sartorius, USA)
### 3.2.5 Evaluation of different chelators

Flasks with 50 ml NB No2 and 10 mM EDTA or 20 mM for the other chelators were prepared. The pH was adjusted to 6.5 and the flasks inoculated with fresh inoculum of *E.coli* in stationary phase, grown in NB No2 at 30°C for 18 hours. The flasks were incubated at 30°C until *E.coli* reached a population where monitoring with a spectrophotometer was possible (~10^7 cells/ml). Different concentrations of nisin were then added and readings of O.D. at 600 nm with a PYE Unicam SP6-450 UV/VIS spectrophotometer taken at regular intervals. All the experiments were carried out at least in duplicate.

### 3.2.6 Addition of chelators in fermented broth.

Fresh inoculum of stationary phase *L. lactis* NCFB 497 from M 17 broth was used to inoculate 10 ml NB No2 containing 1% glucose. After overnight incubation at 30°C, the culture was added at a level of 1% to fresh NB No2 containing 1% glucose to give a population of approximately 10^6 cfu/ml. This culture was then incubated for 10 hours at 30°C. The same procedure was carried out for the non-nisin producer *L. lactis* NCFB 712. The fermented medium was aliquoted in 100 ml volumes. EDTA or pyrophosphates were added to give a concentration of 10 mM and 20 mM respectively. Treatments without chelators were used as controls. Two different pH levels were used; the final pH after 10 hours growth of *L. lactis* (4.0-4.2) and the pH 7 ± 0.1 which was adjusted with concentrated NaOH solution. Freshly prepared inoculum of the stationary phase Gram negative bacteria in NB No2 was added to the media to give a population of 10^4 cfu/ml. The samples were incubated at 30°C. The treatments were carried out at least in duplicates for both *L. lactis* NCFB 497 and 712.

Enumeration was carried out after dilution MRD using 0.1 ml spread plates. The lactic acid bacteria were counted on M17 agar and confirmed by Gram staining of selected colonies. Gram negatives were enumerated on Nutrient Agar, where they could be readily distinguished from any lactic acid bacteria that grew. The pH of the substrate was monitored by using a BDH Gelplus, double junction, flat tip, pH electrode (BDH,
Merck Ltd, Poole, Dorset, UK) and the acidity by titration with NaOH 0.05 N expressed as percentage acidity of lactic acid.

In one experiment the lactic acid bacteria were removed by centrifugation at 1500xg for 10 min and filtration of the supernatant through 0.45 μm filters (Minisart, NML, Sartorious, USA) in order to evaluate the effect of nisin and chelators without the presence of lactic acid bacteria. Experiments were carried out at least in duplicate.

3.3 RESULTS

3.3.1 Inhibition of *Escherichia coli* by nisin and various chelators

The inhibition of *Escherichia coli* ATCC 25922 growth by the combined action of nisin and chelators was monitored by optical density measurement at 600 nm. (Figure 3.1). EDTA was the most effective chelator in combination with nisin. Even at low nisin concentrations, growth of *Escherichia coli* was totally inhibited, since turbidity was not developed. The *E. coli* could have been inactivated but for such an inference plate counts should have been carried out. EDTA in the absence of nisin was also inhibitory to growth, more so than citrate, phosphate or pyrophosphate, since the chelator-only control O.D. remained below 0.6 over 5 hours incubation. In combination with nisin, the inhibition produced by each chelator at pH 6.5 was in the order: EDTA > pyrophosphate > citrate > phosphate.

3.3.2 Growth of *Lactococcus lactis* and nisin production

Both *Lactococcus lactis* strains grew very well in NB No2 supplemented with 1% glucose at 30°C. They showed almost identical growth, pH decrease and acid production (Figure 3.2). After 10 hours of incubation the pH was 4.1 ± 0.1 and the acidity 0.32 ± 0.01 % w/v lactic acid.
Figure 3.1 Effect of different concentrations of nisin and various chelators on inhibition of *E. coli* ATCC 25922 in Nutrient Broth No2 at 30°C. Control (chelator only) ●, 200 IU/ml ○, 500 IU/ml ■, and 1000 IU/ml □. Range bars are shown.
Figure 3.2 (a) Growth of *L. lactis* NCFB 497 •, *L. lactis* NCFB 712 ○, and nisin production ■, by nisin producing strain *L. lactis* NCFB 497.

(b) Acidity development and pH drop of *Lactis* NCFB 497 (■ and ○ respectively) and *L. lactis* NCFB 712 (○ and □ respectively).
The nisin produced by *Lactococcus lactis* NCFB 497 was determined using the nisin bioassay described by Fowler *et al.* (1975). The diameter of the inhibition zone against the logarithm of nisin concentration is a straight line for concentrations up to 20 IU/ml (Figure 3.3). The correlation was very good since $R^2 = 0.99$. *L. lactis* NCFB 497 produced its maximum level of nisin (290 IU/ml) after 8 hours of incubation at 30°C, when it had reached the stationary phase of growth (Figure 3.2).

### 3.3.3 Effect of *in situ* produced nisin and chelators

In broths fermented with the nisin-producing *L. lactis* (pH 4.0) to which *E. coli* and 10mM EDTA were then added, levels of *E. coli* remained relatively constant over the first 16 hours at 30°C but then dropped by more than 3 logs during the following 16 hours. In the absence of EDTA, counts declined more slowly giving an overall reduction over 32 hours of about 1 log (Figure 3.4a). This greater lethal effect in the presence of EDTA was due to the EDTA alone and not a result of it increasing the sensitivity of the *E. coli* to nisin since a similar drop was seen with the non-nisin producing culture which was in all other respects identical (Figure 3.4b). The cell population and acid production of both *L. lactis* NCFB 497 (nisin producer) and 712 (non-nisin producer) were not influenced by the presence of EDTA. During the course of the experiment they continued to produce acid and the pH decreased from 4.0 to 3.7. (Figure 3.5a and b).

Comparing the treatments without EDTA for nisin-producing *L. lactis* NCFB 497 and non-nisin producer *L. lactis* NCFB 712, it can be concluded that the developed lactic acid produced did not render the bacteria more sensitive to nisin.

Adjusting the initial pH of the nisin-containing fermented broths to 7, resulted in a rapid drop of about one log in the *E. coli* population over the first 4 hours when EDTA was present (Figure 3.6a). After that, the decrease in the *E. coli* populations in samples with and without EDTA exhibited similar profiles, with the EDTA treated population dropping below the detection limit of 10 cfu/ml after 32 hours. It is apparent from Figure 3.6a and 3.6b that the one log drop in the presence of EDTA was concurrent with a pH drop from its initial adjusted value of 7.0 to below 4.5 as the *L. lactis* continued to
Figure 3.3 Calibration curve of the diameter of the inhibition zone against the log of nisin concentration. The equation of the line and the $R^2$ is also shown. Standards of 1, 2.5, 5.0, 10 and 20 IU/ml were used. Each point is the average of three replicates.
Figure 3.4 Treatments with (a) nisin producing *L. lactis* NCFB 497 and (b) with non-nisin producing *L. lactis* NCFB 712 with and without 10 mM EDTA (initial pH 4.0). Counts of *L. lactis* without ● and with ○ EDTA and *E. coli* ATCC 25922 without ■ and with EDTA □. The arrow (↓) shows the detection limit. Range bars are shown.
Figure 3.5 Treatments with (a) nisin producing *L. lactis* NCFB 497 and (b) with non-nisin producing *L. lactis* NCFB 712 with and without 10 mM EDTA (initial pH 4.0). pH drop without • and with ○ EDTA and acidity development without ■ and with □ EDTA. Range bars are shown.
Figure 3.6 Treatment with nisin producing *L. lactis* NCFB 497 with and without 10 mM EDTA (initial pH 7.0). The arrow (↓) shows the detection limit. Range bars are shown.

(a) Counts of *L. lactis* NCFB 497 without ● and with ○ EDTA and *E. coli* ATCC 25922 without ■ and with EDTA □.

(b) pH drop without ● and with ○ EDTA and acidity development without ■ and with □ EDTA.
produce acid. This inactivation was not a result of the increased acidity, since it did not occur in the absence of EDTA although the same decrease in pH was seen (Figure 3.6b).

When the non-nisin producing \textit{L.lactis} (NCFB 712) was used, \textit{E.coli} ATCC 25922 surprisingly survived better in the presence of EDTA (Figure 3.7a) than in the control (without EDTA). This can be ascribed to the inhibitory effect the EDTA had on the lactic acid bacteria which decreased in numbers by about 2 logs over 32 hours in the presence of EDTA compared with a 0.5 log drop in its absence. This resulted in less acid development and a smaller pH drop (Figure 3.7b).

Figures 3.8a and b show the results of a treatment with the nisin producer, \textit{L. lactis} NCFB 497, in which the pH was re-adjusted back to 7 after its initial drop to around 4.5. When the pH was increased back to neutrality in the culture containing EDTA, a further decrease in the \textit{E. coli} population was seen. In the absence of EDTA there was a slight increase in numbers of \textit{E. coli} during the experiment as a result of the longer period at neutral or near-neutral pH.

The addition of 20 mM pyrophosphate (the chelator which was most effective after EDTA) did not produce a reduction of \textit{E.coli} population in any of the treatments at any pH (data not shown).

To exclude the effect of acid production by the \textit{L. lactis} during the experiment, the same treatments were conducted after the removal of the lactic acid bacteria. Removal of cells decreased the inhibitory effect since inactivation of \textit{E. coli} at neutral pH was not seen. Growth was however reduced and the combination of EDTA and the \textit{in situ} produced nisin gave a greater inhibition of \textit{E. coli} growth than EDTA alone, which also produced some inhibition (Figure 3.9a). At low pH there was no difference between EDTA and EDTA/nisin action (Figure 3.9b). As before there was no apparent effect when pyrophosphate was used instead of EDTA. Any effect of lactic acid on sensitisation of \textit{E.coli} to nisin was also found to be negligible.

When a cocktail of 5 different \textit{E.coli} strains (\textit{E. coli} 001, 089, 102, 105 and ATCC 25922) was used, the population decrease in the 4 hours taken for the pH to drop was
Figure 3.7 Treatment with non-nisin producing *L. lactis* NCFB 712 with and without 10 mM EDTA (initial pH 7.0). Range bars are shown.

(a) Counts of *L. lactis* NCFB 712 without • and with ○ EDTA and *E.coli* ATCC 25922 without ■ and with EDTA □.

(b) pH drop without • and with ○ EDTA and acidity development without ■ and with □ EDTA.
Figure 3.8 Effect of pH on EDTA / nisin action. Treatment with nisin producing *L. lactis* NCFB 497 with and without 10 mM EDTA where the pH re-adjusted back to the initial value 7.0. The vertical line ( ) indicates when the adjustment of the pH took place. The arrow (↓) shows the detection limit. Range bars are shown.

(a) Counts of *L. lactis* NCFB 497 without ▪ and with ○ EDTA and *E.coli* ATCC 25922 without ■ and with EDTA □.

(b) pH profile without ● and with ○ EDTA.
Figure 3.9 Treatments where the lactic acid bacteria were removed. Counts of *E. coli* in broth fermented by nisin producing *L. lactis* NCFB 497, without chelator •, with 10mM EDTA ■ and with 20mM pyrophosphates ▲, and in broth fermented by non-nisin producing *L. lactis* NCFB 712, without chelator ○, with 10mM EDTA □ and with 20mM pyrophosphates Δ. Range bars are shown.
(a) Initial pH 7.0, and (b) Initial pH 4.0.
0.5 log less than with the *E. coli* ATCC 25922 alone, indicating some variability between strains in this effect (Figure 3.10a). Figure 3.10b shows the effect of a broth fermented by the nisin-producer *L. lactis* 497 and EDTA on the inactivation of *Pseudomonas aeruginosa* and *Salmonella* Enteritidis PT4 when the initial pH was adjusted back to 7. *P. aeruginosa* proved to be more sensitive than *Escherchia coli* in contrast to the salmonellas, which showed no initial decrease attributable to the combined effect of EDTA and nisin. Eventually however, the salmonella did die out more rapidly than the *E. coli* reflecting their lower acid tolerance rather than their sensitivity to nisin/EDTA.

3.4 DISCUSSION

Chelators are compounds able to sequester metal ions and form stable metal complexes. When they chelate Ca$^{2+}$ and Mg$^{2+}$ ions from the outer membrane of Gram negatives they destabilise its structure and alter its permeability (Vaara, 1992) making the cells sensitive to nisin (Stevens *et al.*, 1991; 1992a; Cutter and Siragusa, 1995a). Addition of divalent cations has been shown to diminish the action of nisin (Cutter and Siragusa 1995a; Ganzle *et al.*, 1999). Divalent cations not only stabilise the lipopolysaccharide layer of outer membrane (Nikaido and Vaara 1985) but also bind to anionic phospholipids resulting in an enhanced rigidity of the cytoplasmic membrane and reduced the affinity of nisin to the cytoplasmic membrane (Crandall and Montville 1998).

The stability of metal-chelator complexes is expressed by their pK (log K) (Jeffrey *et al.* 1989) values (the larger the pK the more stable the complex). The respective pKs of the chelation equilibrium for EDTA, pyrophosphate, and citric acid are 10.7, 5.0 and 3.5 for calcium, and 8.7, 5.7 and 2.8 for magnesium (Furia, 1972). These values accord with our results where EDTA, the most effective chelator, gave greatest nisin sensitivity in the *E. coli* test strain, and pyrophosphates were more effective than citrate or phosphates in nutrient broth at pH 6.5. However, because of its high chelating activity, EDTA was also inhibitory for the bacteria in the absence of nisin, since it can also strongly chelate
Figure 3.10 Treatment with nisin producing *L. lactis* NCFB 497 (Initial pH 7.0).

(a) Counts of *E. coli* ATCC 25922 without ■ and with □ 10mM EDTA, and a cocktail of 5 different *E. coli* strains (*E. coli* 001, 089, 102, 105 and ATCC 25922) without ● and with 10mM EDTA○.

(b) Counts of *P. aeruginosa* ATCC 27853 without ◆ and with ◇ 10mM EDTA and *Salmonella* Enteritidis PT4 without ▲ and with △ 10mM EDTA. The arrow (↓) shows the detection limit. Range bars are shown.
metallic trace elements from the medium. EDTA has been found as the most effective chelator in combination with nisin by other researchers as well (Stevens et al., 1992a). Different Gram negative bacteria have different sensitivity to the action of EDTA/nisin. *E. coli* was more sensitive than the salmonellas tested, while the *Pseudomonas* showed greatest sensitivity. This supports the observation of Stevens et al. (1991) who found that an *E. coli* ATCC 25922 population decreased more than *Salmonella Enteritidis Puerto Rico* in treatments with EDTA and nisin in cell buffers.

Both the strains of lactic acid bacteria used grew well in NB No2 supplemented with 1% glucose at 30°C and exhibited almost identical growth rates, pH change and acidity development. The nisin producing strain NCFB 497 produced quite high amounts of nisin (290 IU/ml). At this point the microorganism were in stationary phase having a population of approximately $10^9$ cfu/ml. This is a confirmation of Hurst (1981) that the maximum amount of nisin is produced when the maximal biomass formation has occurred.

The potentiating effect of EDTA on the activity of nisin produced *in situ* against Gram negatives was apparent at neutral and slightly acidic pH but not at acidic pH. Since nisin is active and stable at acidic pH values (Delves-Broughton, 1990), this indicates that the pH is affecting the chelating ability of EDTA rather than nisin activity. Two factors affect the chelation process: the ligand must have the proper steric and electronic configuration in relation to the metal being complexed, and the surrounding milieu (pH, ionic strength, solubility, etc.) must be conducive to complex formation (Furia, 1972). While the ionised carboxylate group of EDTA is an excellent donor group, the non-ionised form is not. The ionisation of the complex is dependent upon the pH of the medium. Lowering the pH increases the proportion of EDTA in its unionised form and decreases the stability of the metal-EDTA complex (Jeffery, et al.1989).

For the chelation of a divalent cation such as $\text{Mg}^{2+}$ by EDTA, the chelation reaction and the stability constant $K$ can be written as:

$$\text{Mg}^{2+} + \text{EDTA}^\ominus \leftrightarrow [\text{EDTA-Mg}]^\ominus$$

$$K = [\text{EDTA-Mg}]^\ominus / [\text{Mg}^{2+}] [\text{EDTA}^\ominus]$$
The apparent, or conditional stability constant at a particular pH, $K_H$, can be calculated from the relationship $K/a$ where $a$ is the fraction of EDTA present as EDTA$^\text{3+}$. Thus $K_H$ can be calculated from the expression:

$$\log K_H = \log K - \log a$$

The factor $a$ can be calculated from the known dissociation constants of EDTA and pH. Log $a$ at pH 5 is 7.0 and log $K$ for Mg is 8.7, thus the apparent stability constant at pH 5 for the Mg-EDTA complex, is 1.7, i.e. the complex has relatively low stability (Jeffery, et al. 1989).

The presence of EDTA in the fermented broth caused a reduction of population and activity (pH drop and acid production) of both $L. lactis$ strains and especially of the non-nisin producing strain NCFB 712. At neutral pH, EDTA, with its high chelating ability, binds trace metals valuable for the fastidious lactic acid bacteria, and causes suppression of their metabolism. At pH 4-4.5 there was not only no inactivation of $E. coli$ due to EDTA but also no suppression of lactic acid bacteria. The effect of pyrophosphate is negligible since its chelating ability is much lower.

While other workers have indicated that nisin production in situ can assist in the inhibition of Gram positive bacteria and may therefore contribute to the safety and stability of fermented foods, the work presented here shows that sensitisation of Gram negatives by chelating agents will not work since the concomitant acid production will antagonise the chelation reaction. The presence of lactic acid produced by the lactic acid bacteria not only inhibited the chelation ability of EDTA but also did not sensitise the Gram-negatives to nisin, for the incubation temperature and time used in these experiments. Kalchayanand et al., (1992) reported that organic acids rendered Gram-negatives sensitive to nisin but after seven days incubation at chill temperature ($4{}^\circ\text{C}$). It is therefore necessary to look at alternative strategies to sensitise Gram negatives to nisin in fermented foods.

Of some interest is the observation that filtration of the broth to remove $Lactococcus$ cells decreased the inhibition of $E. coli$. This suggests that the physical proximity of cells can contribute to the inhibition observed and warrants further investigation.
CHAPTER 4

EFFECT OF NISIN

ON HEAT INJURY AND INACTIVATION OF

SALMONELLA ENTERITIDIS
CHAPTER 4

EFFECT OF NISIN ON HEAT INJURY AND INACTIVATION OF SALMONELLA ENTERITIDIS

Work from this chapter has been published under the title, 'Effect of nisin on heat injury and inactivation of Salmonella enteritidis PT4', in the Int. J. of Food Microbiology 43 (1998) 7-13.

SECTION A: A STUDY IN LABORATORY MEDIA

A 4.1 INTRODUCTION

Nisin is a bacteriocin produced by Lactococcus lactis subsp. lactis, which is active against Gram-positive organisms including bacterial spores. It is not generally active against Gram-negative bacteria, yeasts and fungi. Gram negatives show nisin-sensitivity when their outer membrane permeability is altered (Hancock 1984; Vaara, 1992). Treatment with chelators such as EDTA, citrate and phosphates are able to alter the OM permeability and render the Gram negatives sensitive to nisin (Stevens et al., 1991; 1992a; Cutter and Siragusa, 1995a).

Physical treatments like heating can sublethally injure Gram negatives, by means of outer membrane permeability damage. Tsuchido et al. (1985) have reported the destruction of OM in E. coli by heating and Kalchayanand et al. (1992) found that sublethally injured Gram-negatives produced by heating are sensitive to nisin. It is therefore possible that sub-lethal injury to Gram negatives during pasteurisation processes could induce sensitivity to nisin.

In this section, we report a study on thermal injury and the heat sensitivity of Salmonella Enteritidis in nutrient media, containing nisin. Factors such as the different phage type, which reflects differences in the outer membrane, the duration of the heat inactivation,
the pH of the heating menstruum, the presence of chelators, the subsequent addition of nisin to the heat injured cells, and the impedimetric method to detect injury were studied.

A 4.2 MATERIALS AND METHODS

A 4.2.1 Organisms

Salmonella Enteritidis PT4, P167807 and PT7, P469815 and Micrococcus luteus for the nisin bioassay were used in this study. Details about the microorganisms, their maintenance and resuscitation can be seen in Chapter 2.

A 4.2.2 Chemicals and Microbiological Media

Disodium EDTA, was supplied by Fisons (Fisons, FSA Laboratory Supplies, Loughborough, UK). Concentrated solution was prepared by dissolving the EDTA in deionized water and filter sterilizing through 0.45 µm filters (Miniart, NML, Sartorius, USA). All the microbiological media used were supplied by Oxoid (Basingstoke, Hampshire, UK) unless otherwise stated.

A 4.2.3 Nisin

See General Materials and Methods (Chapter 2).

A 4.2.4 Nisin assay

The activity of nisin during the heating was determined by horizontal agar plate diffusion according to Fowler et al. (1975) bioassay using Micrococcus luteus, as a target.
microorganism (See General Materials and Methods-Chapter 2). Samples, where nisin added in a concentration of 1000 IU/ml were taken at the beginning and the end of the heat treatment, diluted as appropriate in 0.02N HCl and 0.1 ml of each sample transferred into the wells of the assay medium. Samples without nisin were also taken to serve as controls and reveal any effect of the heated and not heated Nutrient Broth on the inhibition zone of the test microorganism. At least 3 replicates for standards, controls and samples were carried out.

A 4.2.5 Heat challenge.

Resuscitated cultures were diluted tenfold in MRD for the inoculation of pre-warmed NB at 37°C (50 or 100 ml) to give an initial suspension of approximately 1 to 10 cfu/ml. All broths were incubated statically at 37°C for 18-20 h and immediately centrifuged (1000xg for 15 min at 20°C). Previous determination of a growth curve under these conditions had shown the culture to be well into the stationary phase at this stage (Appendix 1).

Centrifuged cell pellets were resuspended in 1 ml pre-warmed NB at 37°C. Nisin solution or 0.02 N HCl (0.2 ml) was added to NB already heated at the temperature of 55°C in order to give the required final nisin concentration. The heating menstruum was held at the temperature of 55°C in a plugged 100 ml conical flask in a thermostatted water bath 55°C ± 0.05°C and inoculated with 1 ml of the prepared suspension. The flask contents were stirred via a magnetic follower, propelled by a custom-made 12 V D.C. submersible stirrer operating at 60 rpm to minimise vortex formation. Temperature regulation was provided by a Haake DC-1 circulator heater (Fisons Scientific Equipment, Loughborough, Leicestershire, UK). Heating menstruum temperature was measured using a NAMAS certified probe and digital indicator (Pt 100 probe and Series 268 indicator; Anville Instruments, Camberley, Surrey, UK).

The pH of NB was adjusted to 5 or 9 by using concentrated solutions of HCl or NaOH respectively. Small volumes of concentrated EDTA solutions were added in order to
achieve the required concentrations of 0.125, 0.25 and 0.5 mM and then the pH adjusted back to normal (7.3) with NaOH. The pH of was measured with a BDH Gelplus, double junction, flat tip, electrode (BDH, Merck Ltd, Poole, Dorset, UK).

For the experiment where heat-stressed cells of *Salmonella* Enteritidis PT4 experienced the effect of nisin in NB at 37°C, the following protocol was employed; *S. Enteritidis* PT4 was heat challenged as described previously for 10 min in order to produce injured cells. The cells were centrifuged (1500xg for 15 min at 20°C) washed and resuspended in MRD in order to inoculate 50 ml of NB containing different nisin concentrations. An uninjured population was also used to inoculate NB with and without nisin after it had been diluted in MRD to give similar initial populations. Each treatment was carried out in duplicate.

**A 4.2.6 Enumeration of the cells**

Samples were taken periodically, centrifuged, and the supernatant discarded. The pellet was washed with saline (0.85% NaCl) to remove any remaining nisin, centrifuged again and then resuspended in MRD back to the original volume. Decimal serial dilutions in MRD were enumerated as 0.1 ml spread plates on Nutrient and Xylose Lysine Deoxycholate Agar (NA and XLDA respectively). Plates were incubated for 48 h at 37°C.

**A 4.2.7 Calculations**

The D values were calculated from the linear regression of log survivors against time. The percentage reduction of D value due to nisin compared to the control was also estimated by solving the formula: 

\[
\left( \frac{D_{\text{control}} - D_{\text{nisin}}} D_{\text{control}} \right) \times 100
\]

The percentage of difference between D values derived from Nutrient Agar and XLD Agar (XLDA) counts, compared to the D value derived from NA counts was also estimated by using the formula:

\[
\left( \frac{D_{\text{NA}} - D_{\text{XLDA}}} D_{\text{NA}} \right) \times 100
\]
A 4.2.8 Capacitance measurements

Capacitance measurements were made with the Bactometer Model 120SC (Bactomatic, Inc. USA) with a Model 123 central data processor (bioMerieux). The heat challenge procedure was the same as described previously. Samples were taken every 5 minutes, diluted in MRD to give population less than $10^5$ cfu/ml and 0.1 ml of the diluted samples (in duplicate) was placed into the Bactometer disposable module wells (bioMerieux Vitek, Inc. 99052) containing 1 ml of Bactometer General Purpose Plus Medium (BGPPM, bioMerieux). The detection time, (DT), was recorded after incubation of the Bactometer wells at 37°C. Cells were also enumerated by plating 0.1 ml of the same dilution used to inoculate the Bactometer well, on NA and XLDA and incubating it at 37°C for 48 hours. A calibration graph of log (cfu/ml) on NA against DT was first plotted by using non-heat stressed *Salmonella* Enteritidis cells.

A 4.3 RESULTS

A 4.3.1 Injury and inactivation during heating

Sublethal injury in survivors during heating at 55°C was assessed using two methods: the difference between counts on selective (XLDA) and non-selective (NA) media and the extension of capacitance detection time for survivors relative to that predicted from the calibration curve for the same number of uninjured cells (Alexandrou et al., 1995).

Detection times of heat-treated *Salmonella* Enteritidis PT4 cells compared with the calibration curve for uninjured cells are shown in Figure A 4.1. It can be seen that heat injured cells gave higher detection times than uninjured cells whose detection times are represented by the calibration curve (expected detection time).

The difference in counts on NA and XLDA were also used to detect the injury caused by heating. After 10 min of heating an almost two logarithmic cycles difference was
Figure A.4.1. Effect of heat injury on Detection Times of *Salmonella* Enteritidis PT4. The straight line is the regression line of the expected detection times, taken using uninjured population. O indicates results obtained with injured populations where cells show extended detection times.
apparent (Photo 4.1a and b) in contrast to unstressed culture (Photo 4.2a and b). Colony sizes on plates were more varied for heated cells compared to the uninjured cells. A lot of small colonies were apparent on the plate with the injured suspension (Photo 4.1a) in contrast to the uninjured cells (Photo 4.2a), which gave more uniform colonies indicating that the majority of the survivors were injured cells, requiring time to repair, recover and subsequently grow.

Both methods showed the initial population to be uninjured (zero extension of detection time and identical counts on NA and XLDA), and the extent of injury to increase during heating, as indicated by the increasing differences between the colony counts on XLDA and NA and the predicted and observed detection times (Figure A.4.2). The actual extent of injury can be represented by the extension of detection time and by the difference between counts on non-selective and selective media as a proportion of the total population ((NA-XLDA)/NA) (Figure A.4.3). Extension of detection time (DT_{observed} - DT_{predicted}) and proportion of injury curves both show a similar profile with the extent of injury increasing rapidly so that more than 99% of survivors displayed injury after 10 minutes heating, reflected in an extended lag period (detection time) of about 5 hours (Figure A.4.3). After 15 minutes heating the extent of injury increased at a much slower rate, though the total number of survivors at this stage was of course much less.

A 4.3.2 Effect of nisin

To determine whether the increasing injury during heating affected the nisin sensitivity of the organisms, thermal death curves were produced using nutrient broth (pH 7.3) containing different nisin concentrations. The mean D values determined from counts of total survivors (NA) and uninjured survivors (XLDA) are presented in Figure A.4.4 and Table A.4.1. The D value derived from XLDA counts measures the combined rate of both injury and inactivation during heating and is therefore much shorter than the D value from NA counts which measure primarily the rate of inactivation. The D value (derived from NA) gives the rate of reduction of the total population and the XLDA derived D value gives the rate of reduction of the uninjured population. The difference
Heated suspension of *Salmonella* Enteritidis PT4 in NB at 55°C for 10 min, plated on NA.

Dilution $10^{-3}$

Photograph 1. Plates of heated *Salmonella* Enteritidis PT4 cells in NB for 10 min at 55°C, on (a) NA and (b) XLDA.

There is a two log cycle difference between NA and XLDA counts. Colony sizes on plates are also varied in both agar media.
Healthy suspension of *Salmonella Enteritidis PT4* plated on NA. Dilution $10^{-6}$

Healthy suspension of *Salmonella Enteritidis PT4* plated on XLDA. Dilution $10^{-6}$

**Photograph 2.** Plates of healthy cells of *Salmonella Enteritidis PT4* on (a) NA and (b) XLDA.

There is no difference between counts on NA and XLDA for uninjured suspensions.

Colony sizes are also uniform.
Figure A.4.2. Differences between NA and XLDA counts, and expected (predicted by the calibration graph of the uninjured population—Figure A.4.1) and extended (observed) detection times (DT).

Figure A.4.3. The proportion of injured cells (NA-XLDA/NA) and the increase of the Detection Time.
Figure A.4.4. Effect of nisin on the D value of S. Enteritidis PT4 at 55°C in Nutrient Broth (pH 7.3). The error bars show the standard deviation.

<table>
<thead>
<tr>
<th>Nisin (IU/ml)</th>
<th>Observed D values (min) ± st.dev.</th>
<th>Reduction</th>
<th>D values (min) ± st.dev. reduction (D_{NA} - D_{XLDA})/D_{NA}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>6.32 ± 0.50</td>
<td>0.0</td>
</tr>
<tr>
<td>500</td>
<td>4</td>
<td>5.63 ± 0.26</td>
<td>10.8</td>
</tr>
<tr>
<td>1000</td>
<td>3</td>
<td>5.16 ± 0.21</td>
<td>18.1</td>
</tr>
<tr>
<td>1500</td>
<td>3</td>
<td>4.60 ± 0.34</td>
<td>26.9</td>
</tr>
<tr>
<td>2000</td>
<td>3</td>
<td>4.51 ± 0.16</td>
<td>28.4</td>
</tr>
<tr>
<td>2500</td>
<td>3</td>
<td>4.38 ± 0.14</td>
<td>30.5</td>
</tr>
</tbody>
</table>

Table A.4.1. D values and the percentage of reduction of the D values of S. Enteritidis PT4 at 55°C in NB with and without added nisin.
between those two D values represents the extent of the injury. The higher the difference the greater the extent of sublethal injury in the surviving population. Since the D value is not always the same, the magnitude of decrease of XLDA D value compared to NA derived D value \((D_{NA}-D_{XLDA})/D_{NA}\), expressed for convenience in percentage \([(D_{NA}-D_{XLDA})/D_{NA}]\times100\%\), offers a non-biased parameter to evaluate the level of sublethal injury.

From Figure A.4.4, it is apparent that as nisin concentration increases, the NA-derived D values decrease by up to 30%. However, the XLDA-derived D value decreased only slightly, but significantly \((p<0.05, \text{Table A.4.2})\). This indicates that nisin is killing primarily those cells that have been sub-lethally injured by the heat treatment whereas uninjured cells are less affected.

A saturation effect of nisin against injured cells is also apparent. Concentrations of nisin higher than 1500 IU/ml did not cause any significant additional effect, since analysis of variance showed that D values for \(S.\) Enteritidis PT4 at nisin concentrations of 1500, 2000 and 2500 IU/ml did not differ significantly \((p>0.05, \text{Table A.4.3})\), in contrast with the D values obtained at 0, 500, 1000 and 1500 IU/ml \((p<0.05, \text{Table A.4.4})\).

The \([(D_{NA}-D_{XLDA})/D_{NA}]\times100\%\) value is decreased as nisin concentration increases up to 1500 IU/ml (Figure A.4.4). Given that the NA derived D value decreases with nisin sensitivity, it is apparent that the injured population decreases because the majority of the cells that are injured become inactivated by nisin. The fact that the \([(D_{NA}-D_{XLDA})/D_{NA}]\times100\%\) value is decreased as nisin concentration increases up to 1500 IU/ml and then remains almost constant, further indicates the saturation effect of nisin against the injured \(S.\) Enteritidis cells.

A 4.3.3 Different bacterial strain

Heat challenge of \(Salmonella\) Enteritidis, PT7, in NB at 55°C gave a D value of 4.3 min (Table A.4.5), showing that PT7 strain is more heat sensitive than \(S.\) Enteritidis PT4, \(D\)
<table>
<thead>
<tr>
<th>Groups (nisin IU/ml)</th>
<th>Obs.</th>
<th>Sum</th>
<th>Average</th>
<th>Variance</th>
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<tr>
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ANOVA

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<th>df</th>
<th>F</th>
<th>p</th>
<th>F crit.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>5</td>
<td>3.828</td>
<td>0.019</td>
<td>2.901</td>
</tr>
</tbody>
</table>

Table A.4.2 Comparison of the mean XLDA derived D values of S. Enteritidis PT4 at 55°C in NB. The differences between XLDA-D values taken by heating in presence of different amounts of nisin, are statistically significant (p<0.05)
<table>
<thead>
<tr>
<th>Groups (nisin IU/ml)</th>
<th>Obs.</th>
<th>Sum</th>
<th>Average</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1500</td>
<td>3</td>
<td>13.81</td>
<td>4.60</td>
<td>0.114</td>
</tr>
<tr>
<td>2000</td>
<td>3</td>
<td>13.53</td>
<td>4.41</td>
<td>0.027</td>
</tr>
<tr>
<td>2500</td>
<td>3</td>
<td>13.14</td>
<td>4.38</td>
<td>0.019</td>
</tr>
</tbody>
</table>

### ANOVA

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>df</th>
<th>F</th>
<th>p</th>
<th>F crit.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>2</td>
<td>0.704</td>
<td>0.531</td>
<td>5.143</td>
</tr>
</tbody>
</table>

**Table A.4.3.** Comparison of the mean D values at 55°C of *S. Enteritidis* PT4 in NB, taken by heating in presence of 1500, 2000 and 2500 IU/ml nisin. Analysis of Variances showed that the differences are not statistically significant (p>0.05).

<table>
<thead>
<tr>
<th>Groups (nisin IU/ml)</th>
<th>Obs.</th>
<th>Sum</th>
<th>Average</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>31.62</td>
<td>6.32</td>
<td>0.247</td>
</tr>
<tr>
<td>500</td>
<td>4</td>
<td>22.52</td>
<td>5.63</td>
<td>0.067</td>
</tr>
<tr>
<td>1000</td>
<td>3</td>
<td>15.47</td>
<td>5.16</td>
<td>0.043</td>
</tr>
<tr>
<td>1500</td>
<td>3</td>
<td>13.81</td>
<td>4.60</td>
<td>0.114</td>
</tr>
</tbody>
</table>

### ANOVA

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>df</th>
<th>F</th>
<th>p</th>
<th>F crit.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>3</td>
<td>15.049</td>
<td>0.00033</td>
<td>3.587</td>
</tr>
</tbody>
</table>

**Table A.4.4.** Comparison of the mean D values at 55°C of *S. Enteritidis* PT4 in NB taken by heating in presence of 0, 500, 1000 and 1500 IU/ml nisin. Analysis of Variances showed that the differences are statistically significant (p<0.05).
value = 6.3 min – Table A.4.1). In Figure A.4.5 the effect of nisin on D values is shown. As the nisin concentration increases, the NA-derived D values decrease significantly (Table A.4.6) by up to 26% while the XLDA-derived D value did not decrease significantly (p>0.05) (Table A.4.7). Hence, once again nisin inactivates only cells that have been injured presumably in their outer membrane. However, a saturation effect was not shown for nisin concentration up to 2500 IU/ml.

The percentage reduction of D value caused by different amounts of nisin in the heating menstruum for both PT4 and PT7 can be seen from Tables A.4.1 and A.4.5. Different strains showed different susceptibility to nisin. For low nisin concentration (500IU/ml) the percentage reduction of D value was about 10% for both. Addition of nisin (up to 2500IU/ml) caused a further decrease of the D values reaching approximately, 30% for S. Enteritidis PT4, and 26% for S. Enteritidis PT7, showing that the D value reduction for S. Enteritidis PT4 was slightly higher than PT7.

The \[ \left( \frac{D_{NA} - D_{XLDA}}{D_{NA}} \right) \times 100 \% \] value, for treatments without nisin, was estimated to be 57.4 and 45.4 for PT4 and PT7 respectively, showing that the less extensive injury of PT7 strain was responsible for the lower sensitivity to nisin. Injury and inactivation is a dynamic process. At a given time, there are cells healthy, injured and totally inactivated. According to Ray (1993) if a stress is not removed, sublethally injured cells are killed and uninjured cells become sublethally injured. Hence, as the heat process continues, healthy cells will become injured and a proportion of the previously injured cells will become inactivated. The inactivation of PT7 occurs faster, hence less time is available for nisin to act and inactivate injured cells, since the injury state does not remain for long. In order to examine the possibility that the lower D value reduction, of PT7 strain is due to the shorter time that is available for nisin to act, inactivation of S. Enteritidis PT7 at a lower temperature, 54°C, was carried out. The D value was estimated to be 8.0 min and the \[ \left( \frac{D_{NA} - D_{XLDA}}{D_{NA}} \right) \times 100 \% \] value about 49.3%, (45.4% at 55°C) given that the XLDA D value was 4.1 min (Table A.4.8). When nisin was added the D values reduced but not to a greater extent than at 55°C. Despite the longer time that was available for nisin to act, D values were not decreased more (Figure A.4.6).
**Figure A.4.5.** Effect of nisin on the D value of *Salmonella* Enteritidis PT7 at 55°C in Nutrient Broth (pH 7.3). The error bars show the standard deviation.

<table>
<thead>
<tr>
<th>Nisin (IU/ml)</th>
<th>Obscr. D value (min) ± st. dev. reduction</th>
<th>NA counts</th>
<th>%</th>
<th>D value (min) ± st. dev. reduction</th>
<th>NA counts</th>
<th>%</th>
<th>(D_{NA} - D_{XLD A})/D_{NA}</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.27 ± 0.26</td>
<td>0.0</td>
<td></td>
<td>2.35 ± 0.16</td>
<td>0.0</td>
<td></td>
<td></td>
<td>45.4</td>
</tr>
<tr>
<td>500</td>
<td>3.96 ± 0.10</td>
<td>9.2</td>
<td></td>
<td>2.28 ± 0.26</td>
<td>1.9</td>
<td></td>
<td></td>
<td>41.7</td>
</tr>
<tr>
<td>1500</td>
<td>3.45 ± 0.32</td>
<td>19.2</td>
<td></td>
<td>2.25 ± 0.16</td>
<td>10.0</td>
<td></td>
<td></td>
<td>34.8</td>
</tr>
<tr>
<td>2500</td>
<td>3.19 ± 0.31</td>
<td>25.8</td>
<td></td>
<td>2.26 ± 0.23</td>
<td>15.6</td>
<td></td>
<td></td>
<td>29.3</td>
</tr>
</tbody>
</table>

**Table A.4.5.** D values and the percentage of reduction of the D values of *S.* Enteritidis PT7 at 55°C in NB with and without added nisin.
<table>
<thead>
<tr>
<th>Groups (nisin IU/ml)</th>
<th>Obs.</th>
<th>Sum</th>
<th>Average</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>17.09</td>
<td>4.30</td>
<td>0.067</td>
</tr>
<tr>
<td>500</td>
<td>4</td>
<td>15.60</td>
<td>3.90</td>
<td>0.011</td>
</tr>
<tr>
<td>1500</td>
<td>4</td>
<td>13.80</td>
<td>3.45</td>
<td>0.105</td>
</tr>
<tr>
<td>2500</td>
<td>4</td>
<td>12.78</td>
<td>3.19</td>
<td>0.095</td>
</tr>
</tbody>
</table>

ANOVA

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>df</th>
<th>F</th>
<th>p</th>
<th>F crit.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>3</td>
<td>13.133</td>
<td>0.00042</td>
<td>3.490</td>
</tr>
</tbody>
</table>

Table A.4.6. Comparison of the mean D values at 55°C of *S. Enteritidis* PT7 in NB taken by heating in presence of different amounts of nisin. Analysis of Variances showed that the differences are statistically significant (p<0.05).

<table>
<thead>
<tr>
<th>Groups (nisin IU/ml)</th>
<th>Obs.</th>
<th>Sum</th>
<th>Average</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>7.04</td>
<td>2.35</td>
<td>0.003</td>
</tr>
<tr>
<td>500</td>
<td>3</td>
<td>6.83</td>
<td>2.28</td>
<td>0.233</td>
</tr>
<tr>
<td>1500</td>
<td>3</td>
<td>6.79</td>
<td>2.25</td>
<td>0.168</td>
</tr>
<tr>
<td>2500</td>
<td>3</td>
<td>6.77</td>
<td>2.26</td>
<td>0.005</td>
</tr>
</tbody>
</table>

ANOVA

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>df</th>
<th>F</th>
<th>p</th>
<th>F crit.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>3</td>
<td>0.055</td>
<td>0.982</td>
<td>4.066</td>
</tr>
</tbody>
</table>

Table A.4.7 Comparison of the mean XLDA derived D values of *S. Enteritidis* PT7 at 55°C in NB. The differences between XLDA-D values taken by heating in presence of different amounts of nisin, are statistically significant (p<0.05)
<table>
<thead>
<tr>
<th>Nisin (IU/ml)</th>
<th>Obs/NA counts</th>
<th>D values (min) ± st.dev. reduction (NA counts)</th>
<th>%</th>
<th>D values (min) ± st.dev. reduction (XLDA counts)</th>
<th>%</th>
<th>Reduction (D&lt;sub&gt;NA&lt;/sub&gt;-D&lt;sub&gt;XLDA&lt;/sub&gt;)&lt;sub&gt;/D&lt;sub&gt;NA&lt;/sub&gt;&lt;/sub&gt;</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>8.04 ± 0.75</td>
<td>0.0</td>
<td>4.07 ± 0.52</td>
<td>0.0</td>
<td>49.3</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>3</td>
<td>7.57 ± 0.32</td>
<td>5.8</td>
<td>3.86 ± 0.18</td>
<td>5.3</td>
<td>48.9</td>
<td></td>
</tr>
<tr>
<td>1500</td>
<td>3</td>
<td>6.99 ± 0.38</td>
<td>13.0</td>
<td>3.64 ± 0.85</td>
<td>10.6</td>
<td>47.9</td>
<td></td>
</tr>
<tr>
<td>2500</td>
<td>3</td>
<td>6.02 ± 0.34</td>
<td>25.1</td>
<td>3.52 ± 0.53</td>
<td>13.7</td>
<td>41.6</td>
<td></td>
</tr>
</tbody>
</table>

Table A.4.8 D values and the percentage of reduction of the D values of *S. Enteritidis* PT7 at 54°C in NB with and without added nisin.

Figure A.4.6 Percentage reduction of D value caused by the addition of nisin in *Salmonella Enteritidis* PT4 and PT7 strains that were being heated in NB.
results indicate that the shorter O-chain lipopolysaccharide length of PT7 strain (rough strain) (Chart et al. 1989), does not enhance the damage of OM by heat and the sensitisation of the cells to nisin.

A 4.3.4 Effect of the pH

Heat challenge of Salmonella Enteritidis PT4 at 55°C in NB at pH 5 and 9 with different nisin concentrations (0, 500, 1500, 2500 IU/ml) was carried out and D values compared with those of pH 7.3. The alkaline environment found to be the most hostile, since the D value of S. Enteritidis PT4 was the lowest (0.9 min) compared to acidic (3.0 min) and neutral (6.3 min) conditions (Table A.4.9). The pH also affected the degree of sublethal injury. The $\frac{(D_{\text{NA}} - D_{\text{XLDA}})}{D_{\text{NA}}} \times 100 \%$ value was 48.7, 57.4 and 61.0 for the pH 5, 7.3 and 9 respectively showing again the severity of the alkaline pH.

The pH greatly influenced the action of nisin. The highest reduction occurred at pH 5, while at pH 9 there was no significant change of the D value for any of the nisin concentrations added. The D value reduction for 500, 1500 and 2500 IU/ml at pH 7.3 was about 10, 27 and 30% compared to 20, 40 and 50% at the pH 5, respectively (Figure A.4.7). This shows that despite the higher injury that occurs at higher pH’s, the greater sensitivity to nisin at low pH is due to the nisin, which is more active in acidic environments.

A 4.3.5 Effect of the EDTA addition

The addition of EDTA in low concentrations (0.125, 0.25 and 0.5 mM) caused a significant reduction ($p<0.05$) of both NA and XLDA derived D values. Addition of EDTA though, produced larger increase in sublethal injury than in thermal inactivation. XLDA derived D values were reduced more than NA derived D values since the $\frac{(D_{\text{NA}} - D_{\text{XLDA}})}{D_{\text{NA}}} \times 100 \%$ value becomes higher as EDTA is added (Table A.4.10). This indicates that the presence of EDTA increased the rate of cell injury during the heating.
Figure A.4.7 Effect of the pH on nisin sensitivity of *Salmonella* Enteritidis PT4, being heated in Nutrient Broth at 55°C

<table>
<thead>
<tr>
<th>Nisin (IU/ml)</th>
<th>pH 5.0</th>
<th>pH 7.3</th>
<th>pH 9.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.02 ± 0.05</td>
<td>6.32 ± 0.50</td>
<td>0.90 ± 0.15</td>
</tr>
<tr>
<td>500</td>
<td>2.37 ± 0.05</td>
<td>5.63 ± 0.26</td>
<td>1.00 ± 0.04</td>
</tr>
<tr>
<td>1500</td>
<td>1.81 ± 0.17</td>
<td>4.60 ± 0.34</td>
<td>0.96 ± 0.24</td>
</tr>
<tr>
<td>2500</td>
<td>1.53 ± 0.07</td>
<td>4.38 ± 0.14</td>
<td>0.86 ± 0.23</td>
</tr>
</tbody>
</table>

Table A.4.9 Effect of nisin on D values and percentage reduction of D values of *Salmonella* Enteritidis PT4 heated at 55°C in NB at pH 5, 7.3 and 9.0
<table>
<thead>
<tr>
<th>Nisin (IU/ml)</th>
<th>EDTA (mM)</th>
<th>EDTA (mM)</th>
<th>EDTA (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
<td>0.125</td>
<td>0.25</td>
</tr>
<tr>
<td>0</td>
<td>6.32</td>
<td>6.25</td>
<td>5.28</td>
</tr>
<tr>
<td>500</td>
<td>5.63</td>
<td>4.56</td>
<td>3.26</td>
</tr>
<tr>
<td>1000</td>
<td>5.16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table A.4.10 Effect of EDTA and nisin on injury and D value reduction of *Salmonella* Enteritidis PT4, heated in NB at 55°C.
The addition of EDTA acted synergistically with nisin in the reduction of D value. From Table A.4.10 it can be seen that the D values for 500 IU/ml nisin with any concentration of EDTA are far less than the D values taken for 1000 IU/ml nisin and the D values for double the concentration of EDTA in the absence of nisin.

A 4.3.6 Effect of the subsequent addition of nisin on heat injured cells

Healthy and heat-injured cells of *Salmonella* Enteritidis PT4 were also exposed to nisin in NB at 37°C after injury (Figure A4.8 a and b). The injured cells needed more time (10 h) to reach the stationary phase in contrast to the healthy cells, which needed 8 hours. Injured cells in absence of nisin showed no lag phase, although initial counts on XLDA are lower. A lag phase is only apparent in the presence of nisin. After that period the counts are almost identical. In the presence of nisin the lag period is far longer than without nisin. This is an indication that nisin can delay the recovery of the cells, hence the lag phase is extended. Nisin did not only cause more extended lag phases but also lowered growth rates. Nisin in high concentrations (2500 IU/ml) delayed the growth of healthy cells as well (Figure A.4.8a), this did not happen with the low nisin concentration of 500 IU/ml (Appendix 2a). The effect of nisin on healthy Gram-negative cells is only inhibitory, since nisin could not reduce their population in PBS, even when it was present for 5 days at 37°C (Appendix 2b). The effect of subsequent addition of nisin on injured cells in NB does not seem to be lethal but it is far more inhibitory than on the healthy cells.

A 4.3.7 Effect of heating and pH on nisin activity

It was necessary to examine whether or not nisin remained active during heat treatment at all the different pHs. Nutrient Broth, heated or not, did not give any inhibition zone. The assay of nisin before and after the heat treatment showed that there is no loss of activity during the time span of the heat treatments used at any pH (Table A.4.11).
Figure A.4.8 Effect of subsequent addition of nisin on healthy (a) and thermally injured (b) cells of *Salmonella Enteritidis* PT4 incubated at 37°C in NB.
<table>
<thead>
<tr>
<th></th>
<th>blank</th>
<th>NB sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(no nisin)</td>
<td>(with 1000 IU/ml added nisin)</td>
</tr>
<tr>
<td>before heating</td>
<td>NB pH 5.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>NB pH 7.3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>NB pH 9.0</td>
<td>0</td>
</tr>
<tr>
<td>after heating</td>
<td>NB pH 5.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>NB pH 7.3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>NB pH 9.0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table A.4.11** Nisin concentrations before and after the heat treatments in NB at different pHs. Nisin was added in concentration of 1000 IU/ml before heating. NB did not cause any inhibition zone. It can also be seen that there is no loss of nisin activity due to heating for the time tested in any pH.
Injured cells can repair and grow on non-selective media but may not be able to grow on selective media due to their increased sensitivity to selective agents (Ray, 1979; 1993). Clark and Ordal (1969) and Ray et al., (1971) reported that Salmonella typhimurium and Salmonella anatum cells injured by heating and freeze drying respectively were sensitive to deoxycholate, the selective agent used in Xylose Lysine Deoxycholate Agar (XLDA). The NA-XLDA difference is therefore taken to represent the population that has been subject to injury affecting the cell outer membrane which increases their sensitivity to deoxycholate in XLDA (Vaara, 1992). Structural injury renders the cells sensitive to selective media, in contrast to the metabolic injury, in which the cells become sensitive to minimal media (Ray 1979). Since both kinds of injury can occur, injury may not always render the cells sensitive to a particular selective agent and capacitance monitoring offers an alternative instrumental technique, which measures the extended lag phase while injured populations repair. This is reflected directly in the extended detection time (Mackey and Derrick 1984; Alexandrou et al., 1995). Impedimetric techniques detect a broader range of injury.

The similar profile of injury as assessed by impedimetry and differential counts suggests that the outer membrane was a prime site for thermal injury. The D values derived from counts on selective and non-selective media confirm that nisin is primarily inactivating those cells that have been heat injured, presumably those where injury is to the cell’s outer membrane (OM).

Conformational alterations of the OM proteins and lipopolysaccharides which can take place during heating (Katsui et al. 1982) change the structure of the OM and its permeability (Tsuchido et al. 1985). Under these circumstances nisin can gain access to the plasma membrane where it exerts its effect. The saturation kinetics suggest that the number of sites where this can occur is limited so that increased concentrations of nisin have no additional effect once these sites are occupied. However, the saturation point is
different from microorganism to microorganism, since it was not observed for *S. Enteritidis* PT7 in contrast to PT4 strain.

Both salmonellas showed sensitivity to nisin but this varied with the strain. The shorter length of O-chain of PT7 strain, did not influence the heat induced OM damage and nisin sensitivity. *S. Enteritidis* PT7, despite the shorter O-chains, is not a deep rough strain where lack of the LPS make them very sensitive to hydrophobic compounds (Vaara, 1992; Stevens *et al.*, 1992b). The LPS structure covers the whole OM ‘in a tiled roof pattern’ and the heat can not damage the rough strain more than the smooth PT4 one. Even when slower heat inactivation of PT7 strain was used by applying heating at 54°C, the reduction of D value was not enhanced, showing that inactivation by nisin of thermally injured cells is not a time dependant phenomenon, in contrast to inhibition and inactivation of *Salmonella* species by combination of EDTA and nisin, where extended exposure increased inactivation (Stevens *et al*. 1991).

The pH greatly affected the ability of nisin to reduce D values. Despite the more pronounced injury that the alkaline pH caused to the bacterial cells, the presence of nisin did not affect their inactivation, in contrast to the acidic pH, where nisin was more effective in reducing the D values despite the milder injury that occurred. Hurst, (1981) reported that nisin is more stable and active in low pH and autoclaving of nisin solution at pH 7 resulted in a loss of 90% of its activity compared to 40% at pH 5. Assay of nisin after the heating at 55°C, showed almost full recovery of its activity even at pH 9. It is likely that the mild heating for few minutes at pH 9 did not damage the nisin and the high pH only inhibited its activity. When the pH was reduced back to low values during the assay nisin was active again. On the other hand decrease in pH, especially below 5.5, has been reported to increase the activity of all bacteriocins, including nisin, (Ganzle *et al.*, 1999). For these reason the contribution of nisin to the heating time reduction was greater in acidic than neutral conditions and negligible at alkaline pH.

The addition of EDTA enhanced OM damage, and inactivation by nisin, since the chelators can sequester the divalent cations Mg$^{2+}$ and Ca$^{2+}$ from the adjacent LPS molecules and increase of the OM permeability to hydrophobic compounds like nisin.
Nisin acting simultaneously with the heat treatment contributed to the killing of cells. When nisin was added subsequently to heat-injured cells it did not inactivate the cells, regardless of their sensitivity to deoxycholate, but it did delay their repair. The cell's permeability barrier seems to recover rapidly after the treatment, so those survivors are no longer sensitive to nisin, although recovery is not sufficient to exclude smaller molecules such as deoxycholate. Tsuchido and Takano (1988) demonstrated that thermally injured Escherichia coli cells show prolongation of growth delay when they are added to the heat-treated cells hydrophobic antibacterial compounds such as medium-chain fatty acids, antibiotics and dyes. The results further emphasise the value of simultaneous application of inimical treatments rather than their sequential application. Remarkable is the observation that high nisin concentrations can inhibit and delay the growth of healthy Gram negatives. The inhibitory effect of nisin at high concentrations has been also observed for Escherichia coli (Carneiro de Melo et al., 1996).

Nisin can contribute to a reduction in pasteurisation time, helping to reduce costs, conserve heat sensitive characteristics of a product and help meet consumer demands for safe but less processed foods. Protection of nisin activity by heat and low pH in broths and foods has been reported (Delves-Broughton, 1990). On the other hand, lipids and proteins bind nisin and decrease its activity (Jung et al., 1992). Hence, to achieve the maximum nisin effect on heating time reduction, nisin should ideally be employed in minimal fat foods at low pH. The uses of nisin, with other adjuncts, like chelators and/or in combination with other bacteriocins (Hanlin et al., 1993) to increase activity against Gram negatives is of great interest.
SECTION B: AN APPLIED APPROACH

EFFECT OF NISIN ON D-VALUE OF *SALMONELLA ENTERITIDIS* PT4 IN EGG WHITE AND LIQUID WHOLE EGG

B 4.1 INTRODUCTION

It is possible to use the effect of nisin on heat-injured Gram-negative cells, noted in section A of this chapter, practically in pasteurisation processes. Inclusion of nisin could contribute to a reduction of heating time, helping to reduce costs, conserve heat sensitive characteristics of a product and help meet consumer demands for safe but less processed foods.

Egg products are an obvious potential application. Hen's egg has become synonymous with salmonella during the last ten years and a recent survey in the United States reported 48% of unpasteurized liquid egg samples to be contaminated with salmonella (Hogue *et al.*, 1997). Surface soiling of shell eggs with infected faeces can result in contamination of the contents when the eggs are cracked. However, the cuticle membrane directly beneath the shell, when undamaged, is an effective barrier against the microorganisms. Salmonellas can however be incorporated into the egg contents during its development within the oviducts. The egg white however is not a favourable growth medium for microorganisms, the alkaline pH and antibacterial agents such as lysozyme, the low level of nitrogenous compounds and the lack of iron make for an inhibitory environment (Wilkins and Board, 1989). Given ambient storage (20°C) numbers of salmonella will remain static and it is not until three weeks of storage that the antibiotic properties of albumen begin to deteriorate, permitting multiplication. It is therefore recommended that eggs are consumed within three weeks of laying and stored below ambient in a dry environment (Advisory Committee on the Microbiological Safety of Food, 1993). Undercooking of contaminated eggs presents a risk of salmonellosis. Simulated cooking studies with artificially contaminated eggs have concluded that highly infected eggs would result in the survival of salmonellas during all manner of cooking.
methods, this included boiling for up to four minutes (Humphrey et al. 1989). Commercially made products containing eggs, which are eaten raw or lightly cooked, require the use of pasteurised eggs. Liquid whole egg and egg white are processed at 63-65°C and 55-57°C respectively for not less than 2.5 min, producing a 4 logs or more reduction of Enterobacteriaceae including Salmonella (Mossel et al., 1995). The elimination of salmonella with as low as possible treatment intensity is desirable for sensitive products, like eggs. Heat causes quality changes in the egg components, denature proteins and affect functional properties like foaming and emulsification (Herald and Smith 1988). A less severe treatment would retain the viscosity and prevent denaturation of egg proteins and therefore help retain the functional properties, useful in food technology.

In this section, we report a study on thermal injury and the heat sensitivity of Salmonella Enteritidis PT4 in liquid whole egg and egg white in the presence of nisin.

B 4.2 MATERIALS AND METHODS

B 4.2.1 Organisms

Salmonella Enteritidis PT4, P167807 and Micrococcus luteus for the nisin bioassay, were used in this study. Details about the microorganisms, their maintenance and resuscitation can be seen in Chapter 2.

B 4.2.2 Nisin

(See chapter 2, General Materials and Methods)
B 4.2.3 Assay of nisin

The activity of nisin was determined by horizontal agar plate diffusion according to Fowler et al. (1975) bioassay using *Micrococcus luteus* as test organism (See chapter 2, General Materials and Methods). The assay was carried for nisin in both egg white (EW) and liquid whole egg (LWE). One ml samples were taken at the beginning and the end of the thermal treatment in 8 ml of HCl 0.02N. Nisin can best be released from the proteins by macerating the food. Hence, our samples were acidified with concentrated HCl to pH 2, where nisin is very stable, boiled for 5 min, cooled rapidly, adjusted to 10 ml with HCl 0.02N and centrifuged at 1000xg for 20 min. The aqueous supernatant was filtered through GF/A Whatman glass fibre paper (Whatman International Ltd, Maidstone, Kent, U.K.), diluted as appropriate with 0.02N HCl and 0.1 ml of each sample used in the bioassay. At least 3 replicates for both standards and samples were carried out.

B 4.2.4 Heat challenge

Resuscitated cultures were diluted tenfold in maximum recovery diluent (MRD) for the inoculation of pre-warmed NB at 37°C (50 or 100 ml) to give an initial suspension of approximately 1 to 10 cfu/ml. All broths were incubated statically at 37°C for 18-20 h and immediately centrifuged (1000xg for 15 min at 20°C). Previous determination of a growth curve under these conditions had shown the culture to be well into the stationary phase at this stage.

Locally purchased eggs were washed in a detergent solution and immersed for few seconds in industrial methylated spirit (IMS) to kill the microorganisms on the shell and then allowed to air-dry. They were then broken and the contents (egg white or liquid whole egg) collected in a sterile beaker. Then 25ml samples were transferred into 50ml plastic tubes (Bibby Sterilin Ltd, Staffordshire, UK). The pH of both EW and LWE was measured with a BDH Gelplus, double juction, flat tip, electrode (BDH, Merch Ltd, Poole, Dorset, U.K.).

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Centrifuged cell pellets were resuspended in 1 ml NB pre-warmed at 37°C. Nisin solution or HCl 0.02 N (0.2 ml) was added to 25 ml of EW or LWE in order to give the required final concentration and vortexed for 10 s. One ml of the prepared cell suspension was added and the mixture was vortexed again for 10 s. One ml of the inoculated mixture was placed in each of the 75x12 mm test tubes closed with hydrophobic cotton. They were subsequently placed into water bath at 55°C. Temperature regulation was provided by a Haake DC-1 circulator heater (Fisons Scientific Equipment, Loughborough, Leicestershire, UK). Heating menstruum temperature was measured using NAMAS certified probe and digital indicator (Pt 100 probe and Series 268 indicator; Anville Instruments, Camberley, Surrey, UK). The probe was inserted into the geometric centre of the liquid in a control tube containing 1 ml of uninoculated EW or LWE.

B 4.2.5 Enumeration of the cells

With EW and LWE (every 1 and 3 min respectively) the contents of one of the test tubes was cooled immediately to below 37°C by adding 3 ml of Maximum Recovery Diluent (MRD; 0.85% NaCl, 0.1% bacteriological peptone) at room temperature and the suspension transferred to a sterile universal vial. The test tube was rinsed (3 x 2 ml MRD) and the washings transferred to the sterile universal to give the first dilution. Samples were then diluted as appropriate and plated (0.1ml) on to NA and XLDA medium. Plates were incubated for 48 h at 37°C.

B 4.2.6 Calculation of D values

For LWE, D values were calculated from the linear regression of log survivors against time. With EW, the short duration of the heat process meant that the bacterial population was very low by the time the medium reached the 55°C. It was therefore necessary to take the heating up time into account when calculating the D values. Accordingly D values were derived from the number of pasteurisation units at 55°C (P_{55}) required to produce a 4 log reduction in survivors. In such cases, the D value can be worked out by
dividing the P units by four, the log reduction of the population. One \( P_{55} \) corresponds to
a process equivalent in terms of its lethality to one minute at 55°C. The \( P_{55} \) units were
calculated from the survivor curves and lethal rate curves derived from time-temperature
data taken during the heat treatment. With the \( z \) value of \( S. \) Enteritidis PT4 equal to 4°C
(Humpheson et al., 1998), the lethal rate (\( L_v \)) at any time \( t \) is equal to:

\[
L_v = 10^{\left(\frac{T-55}{6}\right)}
\]

where \( T \) is the temperature at time \( t \).

The area under the \( L_v \) curve between times \( t_1 \) and \( t_2 \) which correspond to 7 log cfu/g and
3 log cfu/g survivors respectively, was calculated by using the Fig.P (Version 2.5, FigP
Software Corp., Durham, NC, USA). Each experiment was carried out at least in
triplicate.

B 4.3 RESULTS

B 4.3.1 Heat injury and inactivation in Egg White and Liquid Whole Egg

The different experimental procedure required when determining survival of \( S. \) Enteritidis PT4 in heated EW meant that \( D \) values were derived from the number of \( P_{55} \)
units required for a reduction from 7 log cfu/ml to 3 log cfu/ml of \( S. \) Enteritidis (see
Materials and Methods).

The surface area under the \( L_v \) curve was calculated from the time \( t_1 \) to \( t_2 \), which
corresponds to the 7 log and 3 log survivors respectively. The times \( t_1 \) and \( t_2 \) were
determined from the equation describing the log-linear death seen after 2min of heating.
Figure B.4.1, gives a typical example of such a graph. A similar exercise was done
with XLDA counts. \( D \) values were worked out by dividing the P units by four, the log
reduction of the population between \( t_1 \) and \( t_2 \). The average \( D \) values and the percentage
of reduction of \( D \) value were computed and shown in Table B.4.1. The \( D \) values against
nisin concentration are plotted in Figure B.4.2. The pH of egg white was found to vary
from 9.0 to 9.3.
Figure B.4.1 Thermal death curve and lethal rate (Lv) curve of *Salmonella* Enteritidis PT4 in Egg White.

The regression lines were drawn taking into account the thermal death curves after the 2nd minute, where they seem to belong in a straight line. From the regression lines, extrapolating to heating time axis from the log reduction 7 and 3, the intervals for the integration of the area under the lethal rate curve were set, leading to the estimation of the Pasteurisation Units at 55°C.
<table>
<thead>
<tr>
<th>Nisin (IU/ml)</th>
<th>Obs.</th>
<th>D values (min) ± st.dev.</th>
<th>reduction</th>
<th>D values (min) ± st.dev.</th>
<th>reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NA counts</td>
<td></td>
<td>XLD counts</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>%</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>6</td>
<td>0.71 ± 0.06</td>
<td>0.0</td>
<td>0.47 ± 0.05</td>
<td>0.0</td>
</tr>
<tr>
<td>500</td>
<td>3</td>
<td>0.72 ± 0.05</td>
<td>0.0</td>
<td>0.47 ± 0.06</td>
<td>1.9</td>
</tr>
<tr>
<td>1500</td>
<td>4</td>
<td>0.59 ± 0.07</td>
<td>17.0</td>
<td>0.42 ± 0.08</td>
<td>10.0</td>
</tr>
<tr>
<td>2500</td>
<td>5</td>
<td>0.52 ± 0.04</td>
<td>26.5</td>
<td>0.34 ± 0.03</td>
<td>15.6</td>
</tr>
</tbody>
</table>

**Table B.4.1.** D values from both NA and XLDA counts of *Salmonella* Enteritidis PT4 in EW and the percentage of their reduction.

![Effect of nisin on D value of S. Enteritidis PT4 in Egg White (pH 9.0-9.3).](image)

The error bars show the standard deviation.

**Figure B.4.2** Effect of nisin on D value of *S. Enteritidis* PT4 in Egg White (pH 9.0-9.3).

The error bars show the standard deviation.
In LWE where, unlike NB, there was an appreciable heating up time (6 min), D values at 55°C were calculated from survivors curves once the temperature had reached 55°C. The linear region of the survivor curve extended over a 5 log reduction in survivors and had an \( r^2 > 0.99 \). Figure B.4.3, gives a typical example of such a graph. The equivalent D values from XLDA counts were also calculated and both are shown in Table B.4.2 and Figure B.4.4 with the reduction of D values for different concentrations of nisin. The pH of LWE was found to vary from 7.5 to 7.8.

D values in LWE were considerably longer than in EW presumably a result of the combined effect of the higher pH in EW (9.0 to 9.3 compared with 7.5 to 7.8 in LWE) and the protective effect of fat in the LWE. The reduction of D value and therefore heating time in the presence of nisin was significant in EW \( (p=0.0002) \) but not in LWE \( (p=0.451) \). The percentage reduction in D value in EW was not however as great as that achieved in nutrient broth; 500 IU/ml showed no reduction and for 1500 and 2500 IU/ml the reduction of D value in EW was 17.7 and 26.5% respectively. Equivalent figures for NB were 26.9 and 30% respectively. This can be ascribed to the lower activity of nisin at the pH of the egg white (9.0-9.3) and possible binding of nisin with egg white proteins which reduced its antibacterial activity.

The D values derived from the XLDA counts, were significantly different \( (p<0.05) \) and decreased more in EW with nisin than in NB with nisin. For example, at 2500 IU/ml the percentage of reduction in NB was only 16% in contrast to 26% in EW. This was not seen in LWE, where the XLDA-derived D values did not change significantly with increasing nisin concentration.

**B 4.3.2 Assay of nisin**

Nisin levels were measured before and after all heat treatments and found to be virtually unchanged. As can be seen from Table B.4.3, egg white and liquid whole egg gave an inhibition zone corresponding to 400-600 IU/ml, indicating that some of the egg
Figure B.4.3 Thermal death curves of *Salmonella* Enteritidis PT4 in Liquid Whole Egg, and the temperature profile.

The regression lines, from which the D values were estimated, drawn taking into account the thermal death curve after the 6th minute, where the temperature of the LWE has reached 55°C.
Table B.4.2 D values from both NA and XLDA counts of *Salmonella* Enteritidis PT4 in LWE and the percentage of their reduction.

<table>
<thead>
<tr>
<th>Nisin (IU/ml)</th>
<th>Obs.</th>
<th>D values (min) ± st.dev.</th>
<th>reduction</th>
<th>D values (min) ± st.dev.</th>
<th>reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(NA counts)</td>
<td>%</td>
<td>(XLD counts)</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>4.28 ± 0.43</td>
<td>0.0</td>
<td>3.24 ± 0.48</td>
<td>0.0</td>
</tr>
<tr>
<td>500</td>
<td>3</td>
<td>4.31 ± 0.32</td>
<td>0.0</td>
<td>3.10 ± 0.35</td>
<td>4.3</td>
</tr>
<tr>
<td>1500</td>
<td>3</td>
<td>4.35 ± 0.18</td>
<td>0.0</td>
<td>3.00 ± 0.31</td>
<td>7.2</td>
</tr>
<tr>
<td>2500</td>
<td>3</td>
<td>3.98 ± 0.28</td>
<td>7.1</td>
<td>3.00 ± 0.32</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Figure B.4.4 Effect of nisin on the D value of *S. Enteritidis* PT4 at 55°C in Liquid Whole Egg (pH 7.5-7.8). The error bars show the standard deviation.
Table B.4.3 Nisin concentrations before and after the heat treatments. Nisin was added in concentration of 1000 IU/ml before heating.

It can be seen that egg produces and appreciable inhibition zone, which corresponds to 400-600 IU/ml, due to the antimicrobials present. It can also be seen that there is no loss of nisin activity due to heating for the time tested.

<table>
<thead>
<tr>
<th></th>
<th>blank (no nisin)</th>
<th>egg sample (with 1000 IU/ml added nisin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>before heating</td>
<td>EW 500</td>
<td>1700</td>
</tr>
<tr>
<td></td>
<td>LWE 600</td>
<td>1800</td>
</tr>
<tr>
<td>after heating</td>
<td>EW 400</td>
<td>1800</td>
</tr>
<tr>
<td></td>
<td>LWE 600</td>
<td>1700</td>
</tr>
</tbody>
</table>
constituents are able to cause inhibition of the test organism. Heating did not cause any significant change to nisin activity or antimicrobial potency of the egg as a whole.

**B 4.4 DISCUSSION**

Egg white (EW) and liquid whole egg (LWE) are two heat sensitive products where a reduction in the time/temperature of pasteurisation or an increased lethality with existing protocols is desirable.

In food systems the effect of nisin is far less pronounced. Binding of nisin by lipids (particularly phospholipids) and protein has been identified as the reason for its decreased activity in other complex food systems such as meat (Delves-Broughton, 1990). Lipids are known to interfere with nisin activity (Daeschel, 1990; Jung et al., 1992) and especially lecithin which antagonises nisin action by forming stable complexes with it (Henning, 1986). The presence of such lipids in LWE was probably the cause of the more pronounced neutralisation seen with this product where concentrations as high as 2500 IU/ml did not give any statistically significant reduction in D value. The higher pH in the egg products, 7.5-7.8 in LWE and 9.0-9.3 in EW, compared with NB (7.3) could also reduce the effect, since action against bacteria is more pronounced at lower pHs (Ganzle et al., 1999). However, the macromolecules can also protect the nisin from heat and pH making it more effective than in simple solutions or broths (Delves-Broughton, 1990). Hence, in NB at pH 9 nisin did not cause any reduction of D value (Chapter 4, Section A) in contrast to egg white at the same pH. An alternative explanation may be that egg white components produce more injury during heating thus increasing nisin sensitivity.

Nisin assay showed that its activity remained virtually unchanged after heating, indicating that heating for few minutes, even at pH 9 did not damage the nisin any binding on food surfaces or loss of its activity due to high pH is reversible.
The observation that nisin caused more cell membrane injury in S. Enteritidis PT4 on heating in EW than in NB is interesting and suggests a synergistic action with egg white components which make cells more sensitive to deoxycholate. Lysozyme and transferrins which are present in egg, are possible candidates. Perhaps nisin itself interacts with the outer membrane allowing lysozyme increased access to the underlying peptidoglycan. Synergistic effects of nisin and lysozyme against Gram positive bacteria have been reported by Monticello (1989), though this has not been reported previously in Gram negatives. Egg contains transferrins (ovotransferrin—Wilkins and Board, 1989), which might be able to chelate cations from the outer membrane increasing the structural damage (Ellison et al., 1988; 1990), as was observed in NB with EDTA, and enhance injury. Both assumptions require further investigation.

The results show that nisin at high concentrations, can contribute to a reduction in pasteurisation time in heat sensitive products. In addition, in products such as egg white, the increased injury in survivors will render them more sensitive to other preservation hurdles that may be present.
CHAPTER 5

EFFECT OF TEMPERATURE SHOCK

ON PERMEABILITY DAMAGE AND SENSITISATION

OF GRAM NEGATIVES TO NISIN
CHAPTER 5

EFFECT OF TEMPERATURE SHOCK ON PERMEABILITY DAMAGE AND SENSITISATION OF GRAM NEGATIVES TO NISIN

SECTION A : A STUDY ON LABORATORY MEDIA

A 5.1 INTRODUCTION

Bacteria exposed to temperatures outside their temperature range for growth, become sublethally injured and are progressively inactivated. The damage caused is both structural and metabolic. (Straka and Stokes 1959; Ray and Speck, 1973; MacLeod and Calcott 1976). Freezing involves not only the effect of low temperature but also the side-effects of this process, like mechanical damage by the ice crystals and high concentration of solutes (Ray and Speck, 1973). Sites that are commonly damaged by chilling, freezing, and heating are the wall and membrane. Leakage of cations and UV absorbing material has been recorded (Russel and Harries 1967; Strange and Ness, 1963; Calcott and MacLeod 1975), as well as damage of the permeability barrier (Strange and Postgate 1964; Ray et al., 1976; Kempler and Ray 1978; Hitchener and Egan 1977; Tsuchido et al, 1985).

Gram negatives show nisin sensitivity when their outer membrane (OM) permeability is altered by chelators or other means (Hancock 1988; Stevens et al. 1991; Vaara, 1992). Heating and freezing have been shown to increase sensitivity to nisin (Kalchayanand et al., 1992; Boziaris et al., 1998). Prolonged chill storage at 6.5°C, has been shown very recently to sensitise the Gram negatives to nisin (Ellisason and Tatini, 1999).
In this section the effect of low temperature shocks like rapid chilling and freezing/thawing on sublethal injury, permeability damage and nisin sensitivity was studied in a number of Gram negatives: *Salmonella* Enteritidis PT4 and PT7, *Escherichia coli*, *Pseudomonas aeruginosa* and *Pseudomonas fragi*. Heating was also studied using a different protocol than that used in Chapter 4, in order to be comparable with the other two treatments.

### 5.2 MATERIALS AND METHODS

#### 5.2.1 Organisms, nisin, microbiological media

*Salmonella* Enteritidis PT4 and PT7, *Escherichia coli*, *Pseudomonas aeruginosa* and *Pseudomonas fragi* were used in this study. (See Chapter 2).

Microbiological media were supplied by Oxoid (Basingstoke, Hampshire, UK), unless otherwise stated. NA supplemented with 0.15 % SDS, was prepared by adding SDS concentrated solution sterilized by filtration through 0.45 μm filters (Minisart, NML, Sartorius, USA), into molten autoclaved NA. The final volume of the agar contained the appropriate amounts of nutrients and SDS.

#### 5.2.2 Chemicals

Sodium dodecyl sulfate (SDS) and 1-N-phenylnaphthylamine (NPN) were provided by Sigma (Sigma-Aldrich Ltd, Pole, Dorset, UK). Solutions of SDS 3% in distilled water were made and sterilised by filtration through 0.45 μm filters (Minisart, NML, Sartorius, USA). Solution of NPN 1mM in acetone was prepared and stored shortly at 4°C.
A 5.2.3 Temperature shock treatment

Chilling, freezing and heating of bacterial populations were conducted in NB in MacCartney’s bottles. A number of treatments were also conducted in PBS. Resuscitated cultures were diluted tenfold in MRD for the inoculation of 50 ml pre-warmed NB at 37°C or 25°C to give an initial suspension of approximately 1-10 cfu/ml. All broths were incubated statically at 37°C or 25°C for 18-24 h and immediately centrifuged (1500 x g for 15 min at 20°C). Previous determination of a growth curve under these conditions had shown the cultures to be in the stationary phase at this stage (Appendix 1). The pellets were washed in saline and resuspended in NB to a cell concentration of about $10^9$-$10^{10}$ cfu/ml. Then 100 µl of the resuspended suspension was added to 9.9 ml NB with or without nisin, to give a population of about $10^7$-$10^8$ cfu/ml.

Freezing shock was carried out by transferring the cell suspension into a freezer at -20°C and leaving for 2 h. The suspensions were then placed in a water bath at 37°C for 10 min, where they were defrosted and the temperature raised to 37°C.

For chilling shock, the vials with the treatment menstruum had been chilled down to 0.5°C, in an iced-water bath. The suspensions were then added and left for 10 min, before placing in a 37°C water bath for 5 min to reach 37°C. The temperature was recorded with a NAMAS certified probe and digital indicator (Pt 100 probe and Series 268 indicator, Anville Instruments, Camberley, Surrey, UK).

For the heating treatment the vials with the suspensions were transferred in a thermostated waterbath (Haake DC-1 circulator heater, Fisons Scientific Equipment, Loughborough, Leicestershire, UK) operating at 55 ± 0.1°C and were left for 10 min. The vials were then removed and cooled down immediately in cold-water at 4°C and placed in a waterbath at 37°C for 5 min to reach 37°C.
A 5.2.4 Treatments with nisin

In the case of treatment in the presence of nisin, 0.1 ml of nisin solution in HCl 0.02 M (pH 2) or HCl acid only (control) had been added to 9.8 ml NB (and PBS in few cases) in MacCartney bottles, before 0.1 ml of concentrated suspension was added to give a maximum volume of 10 ml. In the case of addition of nisin after the treatments, 0.1 ml nisin solution or HCl were added after the temperature stress had been carried out and the suspension was back at 37°C. The final concentration of nisin used in both cases was 0, 50, 500 and 2500 IU/ml.

A 5.2.5 Treatments with SDS

For the SDS experiment, 0.1 ml of the concentrated suspension was added in 9.4 ml NB where 0.5 ml SDS 3% or distilled water (control) had been added to give a final concentration of 0.15 % and a total volume of 10 ml. In the case of subsequent treatment, 0.5 ml SDS or distilled water added in 9.5 ml suspension after the temperature had reached 37°C.

A 5.2.6 Treatments with NPN

For the NPN experiment, 0.1 ml of the concentrated suspension was added in 9.7 ml PBS and 0.1 ml NPN was added before or after the treatment to give a final concentration of 10 μM. Nisin or HCl acid had been added before or added after, depending on the experiment, to give a maximum volume of 10 ml. The fluorescent intensity of the samples was measured at 420 nm following excitation at 350 nm using a Perkin Elmer LS-5 Luminescence Spectrometer. The effect of NPN fluorescence was tried in both NB and PBS. The fluorescence in NB was much reduced compared to PBS, but gave the same profile. Hence, only PBS was chosen for the completion of all trials.
A 5.2.7 Enumeration and evaluation of the injury

After the treatments, 5 ml of the contents of the vials were transferred to 10 ml centrifuge tubes and centrifuged at 1500xg for 15 min. The pellets were washed in 5 ml saline to remove the remaining nisin and centrifuged again. The bacterial pellet was resuspended in 5 ml MRD, serially diluted and plated as 0.1 ml spread plates on Nutrient Agar (NA) and Nutrient Agar supplemented with 0.15% SDS. Salmonella Enteritidis PT4 and PT7 were also plated on Xylose Lysine Deoxycholate Agar (XLDA). The plates were incubated at 37°C or 25°C for 24 to 48 h. The evaluation of injury and inactivation for the subsequent addition of nisin immediately after the treatments, was carried after 10 min for heating and chilling and 30 min after freezing. The same procedure of washing and plating was carried out.

A 5.3 RESULTS

A 5.3.1 The effect of treatments on injury and inactivation

The number of survivors and the extent of the injury were determined by using cultural methods. The extent of the injury was evaluated by comparing the counts on non-selective (NA) medium and selective media (XLDA and NA containing SDS) media. XLDA contains deoxycholate, which is, like SDS, biological membrane disrupter. Cells that are injured on the outer membrane allow these two surface-active compounds to gain access to the plasma membrane and exert their action (Ray 1979). The correct concentration of the SDS in NA was decided following some preliminary trials. The concentration of 0.15% was the highest that did not affected the healthy cells and gave almost identical results with XLDA. Figures A.5.1, 5.2 and 5.3 show the effect of chilling, freezing and heating respectively, both in the presence and the absence of nisin, on bacterial populations. XLDA counts are not shown, since they were similar to NA/SDS counts.
Figure A.5.1. Effect of chilling and nisin on injury and inactivation of *Salmonella Enteritidis* PT4, PT7, *Escherichia coli*, *Pseudomonas aeruginosa* and *Pseudomonas fragi* in NB. The error bars show the standard deviation of at least three replicates. Counts on NA show the total surviving population (uninjured and sublethally injured) and counts on NA/SDS the uninjured population. The difference between them indicates the extent of the injury.
Figure A.5.2. Effect of freezing/thawing and nisin on injury and inactivation of Salmonella Enteritidis PT4, PT7, Escherichia coli, Pseudomonas aeruginosa and Pseudomonas fragi in NB. The error bars show the standard deviation of at least three replicates. Counts on NA show the total surviving population (uninjured and sublethally injured) and counts on NA/SDS the uninjured population. The difference between them indicates the extent of the injury.
Figure A.5.3. Effect of heating and nisin on injury and inactivation of *Salmonella* Enteritidis PT4, PT7, *Escherichia coli*, *Pseudomonas aeruginosa* and *Pseudomonas fragi* in NB. The error bars show the standard deviation of at least three replicates. Counts on NA show the total surviving population (uninjured and sublethally injured) and counts on NA/SDS the uninjured population. The difference between them indicates the extent of the injury.
All the physical treatments used reduced the culturable population. Heating had the greatest effect giving a 1-2 log cycle reduction in culturable numbers. Low temperature treatments generally gave reductions less than 0.5 log cycles with cold shock having the lowest bactericidal effect.

Sublethal injury, as measured by comparing the count reduction on selective medium compared with the non-selective medium, was also greatest in survivors of the heating process where counts on the two media differed by 0.5 to 2 log cycles depending on the organism studied. Chilling and freezing gave far lower differences between NA and NA/SDS counts indicating that less than 50% of the survivors were injured. Counts on XLDA for the two salmonellas were similar to those on NA/SDS (data not shown).

A 5.3.2 The effect of nisin on temperature shocked bacteria

When nisin was present in the medium during the different treatments, a dose dependent increase in lethality was observed in most cases (Figures A.5.1, 5.2, 5.3). With the exception of E. coli, increased lethality was most apparent in the chilling and freezing protocols. Ps. aeruginosa was very sensitive to chilling and freezing in the presence of nisin (2-3 log reduction), but not to heating. Salmonella Enteritidis Phage Type 7 was far more sensitive to freezing in the presence of nisin than Phage Type 4. Unstressed microorganisms were insensitive to nisin presence (data not shown), which was expected since it is unable to injure or inactivate healthy Gram negatives (Appendix 2).

The presence of nisin during physical treatment reduced counts on both NA and NA/SDS but to different extents. Heating with nisin reduced the NA counts (total population of survivors) more than the NA/SDS counts (uninjured survivors), showing that nisin inactivated primarily the injured cells, as noted in chapter 4. In freezing and chilling, however, where the population of injured survivors was far less, the addition of nisin still reduced the counts on NA and NA/SDS, but to a similar extent, showing that cells that were not recorded as injured by plating on NA/SDS were inactivated. The same profile for the two salmonellas was taken with XLDA counts (data not shown).
The addition of nisin in low or high concentrations after the treatments did not affect or affected bacterial survival far less (Figure A.5.4, 5.5 and 5.6). *Pseudomonas aeruginosa* was slightly sensitive to high nisin concentrations (2500 IU/ml) added after chilling and freezing, in contrast to salmonellas, which were not sensitive at all. Similar results were seen with the treatments carried out in PBS. Treated cells experienced greater reduction with the nisin present during treatments than when it was added after the treatments (data not shown).

A 5.3.3 The effect of SDS

SDS is an outer membrane permeability probe, which inactivates cells when it reaches their cytoplasmic membrane. In contrast to the previous experiment, where the SDS affected the cells when they were plated onto the NA, here the microorganisms experienced the effect of 0.15% SDS, in the NB, during and after the treatments. Sensitivity to SDS during treatment followed by resistance after, would indicate rapid restoration or recovery of the OM permeability barrier.

The effect of SDS on *Salmonella* Enteritidis PT4, PT7 and *Pseudomonas aeruginosa* population reduction is shown in Figures A.5.7, 5.8 and 5.9. Addition of SDS to unstressed suspensions did not have any effect (data not shown). However, its presence during the treatments affected the populations of the bacteria, especially during freezing and heating.

Heating in the presence of SDS decreased the population of bacteria by 4-5 log cycles, far more than nisin (Figure A.5.9). On the other hand, its presence after the treatments had no or only a slight effect on numbers of survivors.

Presence of SDS during freezing, like nisin, affected mainly *S. Enteritidis* PT7 and *Ps. aeruginosa*, but to a lower extent that nisin. The population reduction for these two strains was one to one and a half log cycles, compared to the two log cycles reduction.
Figure A.5.4. Effect of chilling and nisin addition after the treatment on injury and inactivation of *S. Enteritidis* PT4, PT7 and *Pseudomonas aeruginosa* in NB. The error bars show the standard deviation of three replicates. Counts on NA show the total surviving population (uninjured and sublethally injured) and counts on NA/SDS the uninjured population. The difference between them indicates the extent of the injury.
Figure A.5.5. Effect of freezing/thawing and nisin addition after the treatment on injury and inactivation of *S. Enteritidis* PT4, PT7 and *Pseudomonas aeruginosa* in NB. The error bars show the standard deviation of three replicates. Counts on NA show the total surviving population (uninjured and sublethally injured) and counts on NA/SDS the uninjured population. The difference between them indicates the extent of the injury.
Figure A.5.6. Effect of heating and nisin addition after the treatment on injury and inactivation of *S. Enteritidis* PT4, PT7 and *Pseudomonas aeruginosa* in NB. The error bars show the standard deviation of three replicates. Counts on NA show the total surviving population (uninjured and sublethally injured) and counts on NA/SDS the uninjured population. The difference between them indicates the extent of the injury.
Figure A.5.7. Effect of chilling and SDS presence during (1) and after (2) the treatment in NB on inactivation of *S. Enteritidis* PT4, PT7 and *Pseudomonas aeruginosa*. The error bars show the standard deviation of three replicates.
Figure A.5.8. Effect of freezing/thawing and SDS presence during (1) and after (2) the treatment in NB on inactivation of *S. Enteritidis* PT4, PT7 and *Pseudomonas aeruginosa*. The error bars show the standard deviation of three replicates.
Figure A.5.9. Effect of heating and SDS presence during (1) and after (2) the treatment in NB on inactivation of S. Enteritidis PT4, PT7 and Pseudomonas aeruginosa. The error bars show the standard deviation of three replicates.
that nisin caused. *Salmonella* Enteritidis PT4 was very slightly affected, as with nisin and SDS addition after the treatments left the cells almost unaffected.

Chilling and SDS did not reduce the culturable numbers of salmonellas, as nisin did, and *Ps. aeruginosa* was far less affected. Only a half log reduction was attributed to the presence of SDS, nisin caused about two cycle reduction.

It was generally observed that with the simultaneous addition of SDS, cells were sensitive to that agent in contrast to the subsequent addition, confirming that OM permeability recovered after the treatments. The subsequent addition gave a reduction of culturable numbers very similar to the difference between NA and NA/SDS counts of the treated cells. SDS was more effective than nisin with heat stressed cells, less effective against freeze stressed, and not effective at all against chill-stressed Gram negatives.

### A 5.3.4 Effect of stress and presence of nisin on NPN fluorescence

1-N-phenylnaphthylamine (NPN), is a fluorescent probe for outer membrane permeability. NPN fluoresces when in a hydrophobic environment e.g. the cytoplasmic membrane. When the permeability of the outer membrane is altered, NPN can gain access to the cell membrane and fluoresce (Tsuhido *et al.*, 1989).

NPN was added to cells that were unstressed, physically stressed and physically stressed in the presence of nisin. The excitation wavelength was at 350nm and the emission was scanned from 380 to 520nm. A typical graph is shown in Figure A.5.10 To evaluate of the fluorescence intensity, the change in the maximum at 420nm was taken. The results taken are shown in Table A.5.1.

The fluorescence intensity increased during physical treatment confirming OM damage. When nisin was included the increase in fluorescence during treatment was even more marked in cases where addition of nisin affected significantly the survival of the cells. For example, for heating with 50 IU/ml nisin the fluorescence intensity was not increased, as the population was not reduced by the nisin. Addition of 2500 IU/ml affected not only
Figure A.5.10. Typical graph of stress and presence of nisin on NPN fluorescence.

(1) uninjured cells and NPN, (2) uninjured cells, NPN and nisin
(3) chill stressed cells and NPN, (4) chilled stressed cells, NPN and nisin
Fluorescence intensity (Arbitrary Units)
(excitation 350nm, emission 420nm)

<table>
<thead>
<tr>
<th>Control (no nisin)</th>
<th>S. Enteritidis PT4</th>
<th>S. Enteritidis PT7</th>
<th>Ps. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.0</td>
<td>0.0</td>
</tr>
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<td>6.7</td>
<td>4.0</td>
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<td>15.5</td>
<td>25.5</td>
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</table>

(1) NPN presence during the treatment
(2) NPN presence after the treatment

Table A.5.1. Effect of stress and presence of nisin on NPN fluorescence
the death of the population but also the intensity of the fluorescence. This effect was apparent when nisin and NPN were added to untreated cells but not to such an extent. When NPN was added after treatment, the fluorescence intensity remained still higher than the untreated control indicating that molecules such as NPN could still gain access to the cytoplasmic membrane.

A 5.4 DISCUSSION

The viability of microorganisms was affected by the treatment to different extents for the different microorganisms. This was expected since different bacteria exhibit different sensitivity to various factors. A large percentage of the survivors were sublethally injured as indicated by their inability to form colonies on NA supplemented with SDS. The inability of the cells to form colonies on NA with SDS declares structural injury, especially related with the permeability barrier (Ray and Speck, 1973; Ray 1979). The heated cells were more severely damaged than chill- and freeze-injured cells, showing the different effect of each treatment. Indeed, the process itself, the microorganism and other factors related to the suspending medium, the medium used to assess viability, the selective compound used to detect injury etc., all affect the susceptibility of microorganisms on injury and inactivation (MacLeod and Calcott 1976). For example, in a different freezing process, 90% of *Salmonella* sp. survivors were unable to grow on XLDA (Ray 1979), compared to maximum 50% in the protocol used in our study.

The presence of nisin during physical treatment reduced counts on both NA and NA/SDS but to different extents. Heating with nisin reduced the NA counts (total population of survivors) more than the NA/SDS counts (uninjured population) indicating that organisms, which were sublethally damaged during heating, had been inactivated by the nisin during heating. In freezing and chilling, however, where the population of injured survivors was far less, the presence of nisin during treatment reduced the counts on NA and NA/SDS to a similar extent. Cells that survived the physical treatment were insensitive to nisin added after treatment regardless of whether or not they displayed any injury (sensitivity to SDS in nutrient agar). This suggests that transient injury during
processing had made the cells nisin sensitive and that all cells injured in this way had either been inactivated by nisin or had recovered rapidly after treatment, hence the differences between counts on NA and NA/SDS were not great.

To support the theory of transient susceptibility due to rapid restoration of the OM permeability barrier after the treatment, the interpretation of the SDS experiment results is necessary. Presence of SDS in the treating menstruum, simultaneously and subsequent to the treatments showed that the OM permeability barrier, in the case of freezing and heating, recovers rapidly after the treatments. This indicates that the OM permeability becomes transiently altered during these two treatments, making the cells sensitive to compounds like nisin or SDS. This alteration is reversible, since the cells are not sensitive to both nisin and SDS added after the treatments, at least to the same extent. In freezing, cells were less sensitive to SDS than to nisin. In heating, only, SDS found to be more effective than nisin in inactivating injured Gram negatives. Considering the sizes of the two molecules, it is expected that SDS would be much more effective in penetrating a damaged OM than nisin and can penetrate the membrane, which has been damaged to a lesser extent. It can also be assumed that nisin must be more effective in causing damage of cell membrane than SDS, hence, even though less nisin penetrates the OM, this low amount is more effective than the higher amount of SDS.

However, no chilling sensitivity to SDS was seen, during or after the treatment, but the cells were sensitive to nisin when it was present during the treatments. This could be an experimental artefact. SDS solidifies at low temperature, hence is not available for action. For this reason, SDS presence during chilling did not cause any effect. In the case of freezing, the temperature is not dropped instantly, hence SDS took its time to cause an effect, compare to the negligible effect in the case of chilling.

The fluorescence of NPN added into the suspension, simultaneously to the treatments, indicated that there is an increase in permeability. However, the same intensity was seen when it was added after the treatment, showing that there is no recovery or that a small molecule like NPN could still gain access, after an incomplete recovery. Indeed, Tsuhido et al., (1989) in a study with heated *Escherichia coli* and NPN found that after the heat
treatment, NPN could still permeate the cell with decreasing rates with time and after 70 min of incubation the rate of permeation was similar to that of untreated cells.

Remarkable was the fact that the fluorescence was in many cases higher in the presence of nisin, where the nisin affected the viability of the cells. This can be attributed to the fact that, when the cytoplasmic membrane is de-energised, by the action of nisin, an increase of outer membrane permeability occurs, hence more NPN is associated with the hydrophobic environment of the membrane, resulting in a higher intensity. This aspect was reported by Helgerson and Cramer (1977). They found that, de-energization of cells results in increased fluorescence of hydrophobic probes and stated the existence of dynamic structural changes which occur in the outer membrane concomitantly with de-energization of the inner membrane and which affect outer membrane function. This aspect was confirmed by other researchers and all data implies that either de-energization of cells results in a marked structural change in the outer membrane, or there is an energized secretion mechanism for hydrophobic compounds in untreated wild-type cells (Hancock, 1984). It is now known that OM allows the penetration of such compounds with slow rates and the apparent impermeability observed is due to the active efflux (Hancock 1997). De-energization of cells causes inhibition of the active efflux processes, thus more NPN remains and gives higher intensities (Nikaido 1996).

Another less probable interpretation of the results is that nisin acts synergistically with the treatment causing the increase of OM permeability, and gave NPN more access to the cytoplasmic membrane. This could be why SDS was not detrimental to chill-stressed Gram-negatives compared to nisin, as nisin synergistically with the low temperature shocks caused outer membrane damage, with concomitant inactivation of the cell. The same opinion could partially support freezing results as well. Self-promoted uptake (Hancock 1997) has been observed for polymyxins and other polycationic antimicrobials. Polymyxins are pentacationic amphipathic lipopeptide antibiotics, characterized by a heptapeptide ring and a fatty acid tail. The polymyxins are bactericidal even against healthy Gram negative bacteria by a dual mechanism of action. Polymyxins bind to the OM and permeabilize it (sublethal action). This allows it to enter the cytoplasmic membrane where it causes leakage of cytoplasmic components (lethal action) (Vaara
The nisin structure is different from polymyxin. However, it is a polycationic amphipathic peptide (Hurst 1981), has the same action site and similar mode of action. Nevertheless nisin is not able to cause any damage on healthy Gram negatives (Vaara 1992), even after prolonged incubation Gram-negatives did not become sensitive to deoxycholate or sodium dodecyl sulphate (Appendix 2), but cells, which have suffered conformational alteration of outer membrane, might do. However, this requires further investigation.

It seems, so far, that the transient susceptibility to nisin is more probable than the synergistic effect with the chilling process, since such an action for the lantibiotics has never been reported, in contrast to the transient susceptibility of Gram negatives to nisin, injured in OM by High Pressure (Hauben et al., 1996).

Temperature shocked cells were sensitive to nisin to different extents for different microorganisms and different physical processes. Treatments like heating and freezing have already been found to render Gram negatives sensitive to the action of nisin (Present Thesis Chapter 4; Kalchayanand et al., 1992). Prolonged chill storage at 6.5°C, has been shown very recently that sensitise the Gram negatives to nisin (Ellason and Tatini, 1999).

Gram negatives are resistant to hydrophobic molecules like nisin due to the outer membrane barrier. Disruption of this barrier by alteration in OM structure and conformation leads to an induced sensitivity (Hancock, 1984; Vaara 1992). Heating is known to cause damage of OM by releasing the lipopolysaccharides (LPS), (Hitchener and Egan 1977; Tsuchido et al., 1985). Vesicles containing LPS and OM proteins are released because of heating (Katsui et al., 1982). Structural and conformational alterations of the OM, which are mainly reversible, have been reported for freezing (Ray et al., 1976; Kempler and Ray, 1978). The damage was located in the heptose phosphate region (Kempler and Ray, 1978). Hence, in these cases, the nisin molecule can penetrate a damaged or altered OM and find its action site, the cytoplasmic membrane.
Chilling alters OM permeability by allowing the influx of compounds that usually are unable to penetrate the cell (MacLeod and Calcott 1976). The kind of damage or alteration the chilling caused was not described. Leader (1972) reported that cold shock causes crystallisation of the liquid-like lipids within the membrane creating channels. When the chilling is slow, the relative mobility of the lipid chains permits them to rearrange and maintain the integrity of the membrane. The temperature of the phase transition varies with the degree of unsaturation and the chain length of the fatty acids (Steim et al., 1969). Haest et al., (1972), reported that the permeability of the cells is strongly dependent on the fatty acid composition of the membrane lipids. Rapid cooling below certain temperatures depending on the fatty acid composition, caused fragility of the cells and was explained as being a consequence of the solidification of the hydrocarbon core. These scenarios could explain the sensitivity of cold shocked Gram negatives to nisin. The OM consists of an asymmetric bilayer, where the LPS occupies the upper layer and phospholipids the layer below (Nikaido and Vaara 1985). Rapid solidification of a fluid membrane can cause cracks and holes and also the anchoring of LPS and OM proteins could become looser. Under these circumstances, nisin can gain access into the cytoplasmic membrane and inactivate the cell.

Different injured microorganisms exhibited different sensitivity to nisin. This variability was not unexpected, since nisin is only likely to inactivate Gram negatives which have suffered sufficient damage to their outer membrane to allow the nisin molecule to pass and this will vary with differences in treatments and in OM composition. Despite the similarities of the OM assembly, differences in the LPS chain length or the amounts of OM proteins on OM of each strain, and the fact that different treatments cause different kinds of damage, explain the variable sensitivity to the treatment/nisin action. The cell membrane composition also affects the resistance to nisin. Resistant mutants of *Listeria monocytogenes* were found to have fatty acids with a higher transition temperature, higher percentage of straight-chain and lower percentage of branched-chain fatty acids (Ming and Daeschel, 1993). Crandall and Montville, (1998), reported that nisin resistant strains of *Listeria monocytogenes* contain significantly more zwitterionic (phosphatidylethanolamine) and less anionic (phosphatidylglycerol and cardiolipin) phospholipids. Also the different ability of nisin to bind on the cell surface possibly affect
the sensitivity. Bhunia et al. (1991) reported that the sensitivity of Gram-positives to bacteriocins compared to Gram-negatives was due to the high binding of bacteriocins on Gram-positive cells surface. Davies & Adams (1994) and Ming & Daeschel (1995) found that nisin-resistant cells of *Listeria monocytogenes* bind less nisin than the sensitive ones. Hence, even if the differences of OM not affect the sensitivity, the endogenous resistance of cell membrane to the pore forming ability of nisin will give rise to differences in sensitivity.

PT4 and PT7 strains differ in the LPS chain length, with PT7 possessing shorter chains (Chart et al., 1989). Heating and chilling rendered them almost equally sensitive to the presence of nisin, in contrast to freezing with nisin which differentiated the two strains in terms of sensitivity. It could be that the ‘hairy’ coverage of the OM by the LPS chains protected cells of PT4 strain from the mechanical pressure of ice crystals on OM. Bennett et al., (1981) reported that variations in the LPS chain length affected the sensitivity of freeze-thaw injured *Salmonella typhimurium* to novobiocin, polymyxin B and SDS.

Summarising, heating, chilling and freezing treatments cause damage of OM and rendered Gram-negatives transiently sensitive to nisin, to different extents for different bacteria and treatments. It seems that the transient susceptibility is related to a recovery or restoration of the outer membrane barrier, when the stress factor is removed, which exclude molecules like nisin but still allow smaller molecules like NPN to pass through.
SECTION B : AN APPLIED APPROACH

DECONTAMINATION OF CHICKEN CARCASSES BY COMBINATIONS OF COLD SHOCK, EDTA AND NISIN

B 5.1 INTRODUCTION

According to the results of the previous section, rapid chilling in the presence of nisin can give a 50-99% decrease in a bacterial population suspended in NB, with Enterobacteriaceae and *Pseudomonas aeruginosa* the most resistant and most sensitive respectively. Chilling transiently alters the OM permeability of Gram negative allowing nisin to gain access to the cell membrane and inactivate the cells.

A practical application of rapid chilling in the presence of nisin could be the decontamination of chicken carcasses, since chilling by immersing the carcass into cold water is a practice after slaughter (Bolder, 1998) which aims to wash the surface and also reduce the temperature of the carcass.

Decontamination of carcasses using various food grade chemicals and their combination has already been tried. Organic acids, especially lactic acid, are effective decontamination agents, according to Smulders *et al.* (1986). Other chemicals like trisodium phosphate (Lillard 1994; Hwang and Beuchat, 1994), various polyphosphates and NaOH (Hwang and Beuchat, 1994) have also been tried, without or in combination with nisin and/or lysozyme (Carneiro de Melo *et al.*, 1997).

Nisin has been tried in decontamination of carcass surfaces especially in combination with food grade chelators (EDTA), with contradictory results. Cutter and Siragusa (1995b) did not find any significant reduction of *Salmonella typhimurium* and *Escherichia coli* attached on lean beef tissue, in contrast to Shefet *et al.*, (1995) who found a significant reduction of *Salmonella typhimurium* attached on broiler carcasses, but less than that occurring in buffers.
In this section, the effect of chilling, EDTA and nisin, individually and in combination on decontamination of chicken carcasses surface was investigated

**B 5.2 MATERIALS AND METHODS**

**B 5.2.1 Organisms and nisin**

*Salmonella Enteritidis* phage type 4 P167807, and PT7 P469815/0 and *Pseudomonas aeruginosa* USCC 2186 were used. Details about the microorganisms, maintenance and resuscitation and the nisin used are given in Chapter 2.

**B 5.2.2 EDTA**

Disodium EDTA, was supplied by Fisons (Fisons, FSA Laboratory Supplies, Loughborough, UK). Concentrated solution was prepared by dissolving the appropriate amount of the chelator in deionized water and sterilizing by filtration through 0.45 μm filters (Minisart, NML, Sartorius, USA).

**B 5.2.3 Treatments in PBS**

Chilling and EDTA treatments and combinations with and without nisin were carried out in PBS for *Salmonella Enteritidis* PT4, PT7 and *Pseudomonas aeruginosa*. The chilling was conducted according to the protocol in section A. EDTA and nisin were added in a final concentration of 20mM and 500 IU/ml respectively. Plating on both NA and NA supplemented with 0.15% SDS, in order to monitor the injury, was carried out.
5.2.4 Decontamination of chicken skin

In order to evaluate the effect of cold shock on decontamination of chicken carcasses from *Salmonella* Enteritidis PT4 the following protocol was used:

Whole chicken was purchased from the local supermarket and the skin was removed carefully. Round pieces, with a surface area approximately 2 cm$^2$, were cut using a sharp cork borer. The pieces were transferred to open sterile Petri dishes, placed under an Ultra Violet lamp, irradiating at a maximum wavelength of 254 nm, and left for 10 min. The pieces inverted and left for 10 more minutes. Then they were stored at -20°C.

The skin pieces were defrosted at room temperature before the experiment.

Overnight (18-24 h) stationary phase culture of *Salmonella* Enteritidis PT4, PT7 and *Pseudomonas aeruginosa*, grown in 10ml NB at 37°C were centrifuged at 1500g for 10 min and the pellet washed once in saline (0.85% NaCl). Centrifuged again (1500g/10min) and the pellet resuspended in 5ml saline.

From the concentrated suspension prepared in saline, 1ml was diluted into 9ml MRD containing 0.01% Tween 20. The population of the final suspension was approximately 10$^6$ cfu/ml. 20 μl were spread on each chicken piece and left to dry for 30 min. The Tween 20 assisted the dispersion of the 20μl droplet on the skin piece. The pieces with the suspension were left to dry at room temperature.

Universal bottles containing tap water were prepared as follows: Control (tap water only), nisin 500IU/ml, EDTA 20mM, nisin and EDTA (500IU/ml and 20mM respectively) were placed in a 37°C water-bath. The same set was prepared and chilled down to 0.5°C in an iced-water bath. The pH was adjusted to 7.0 with concentrated NaOH. Pieces of chicken skin were picked with sterile forceps and then immersed in one of the solutions in tap water and left for 10 min. After 10 min the temperature of the chilled suspensions was raised to 37°C by transferring to a 37°C water bath and left for 5 min. Then, each piece was removed and placed into a sterile stomacher bag with 20 ml of saline. They were stomached for 2 min at medium speed in a Stomacher (Seward medical Ltd, London UK). Decimal serial dilution series prepared in MRD and 0.1 ml plated on NA. The results expressed as cfu per cm$^2$ of skin surface.
The same experiment using *Pseudomonas aeruginosa* was also conducted.

Experiments were designed to minimise variances due to the different batches of cultures. More specifically, for the evaluation of the population reduction, due to the treatments, for each strain, one volume (batch) of microorganism was grown, for each series of replicates, centrifuged, washed and resuspended to give a concentrated suspension. Specific volumes were added to the treating media, and the viable population was measured. Control samples of all kinds were prepared. All the trials were carried out at least in triplicate.

**B 5.3 RESULTS**

The effect of chilling with nisin, EDTA and nisin and the combination of all three, was firstly evaluated in simple buffer system (Phosphate buffered saline, PBS). Then, trials of decontamination of chicken skin from *Salmonella Enteritidis* PT4, mainly and *Pseudomonas aeruginosa* were conducted.

**B 5.3.1 Treatments in PBS**

The results of the treatments in PBS for *Salmonella Enteritidis* PT4, PT7 and *Pseudomonas aeruginosa*, are shown in Figure B.5.1, B.5.2, and B.5.3 respectively.

As can be seen from Figure B.5.1, and B.5.2, at room temperature, not only the nisin and the EDTA, but also its combination, did not affect the population of salmonella, in terms of population reduction or injury, since, both counts on NA and NA/SDS were the same as the untreated control. However, for *Pseudomonas aeruginosa*, 99% of the population were found to be injured (2 log difference between counts on NA and NA/SDS) when treated with EDTA. In the additional presence of nisin the total population was reduced by one log although the proportion of uninjured cells remained similar (Figure B 5.3). Nisin caused not only reduction of culturable population, but also enhanced the injury, since the counts on NA/SDS were further reduced.
Figure B.5.1. Effect of combinations of chilling, EDTA (20mM) and nisin (500 IU/ml) on injury and inactivation of *Salmonella* Enteritidis PT4 in PBS. The error bars show the standard deviation of three replicates. Counts on NA show the total surviving population (uninjured and sublethally injured) and counts on NA/SDS the uninjured population. The difference between them indicates the extension of the injury.
Figure B.5.2. Effect of combinations of chilling, EDTA (20mM) and nisin (500 IU/ml) on injury and inactivation of *Salmonella* Enteritidis PT7 in PBS. The error bars show the standard deviation of three replicates. Counts on NA show the total surviving population (uninjured and sublethally injured) and counts on NA/SDS the uninjured population. The difference between them indicates the extension of the injury.
Figure B.5.3. Effect of combinations of chilling, EDTA (20mM) and nisin (500 IU/ml) on injury and inactivation of *Pseudomonas aeruginosa* in PBS. The error bars show the standard deviation of three replicates. Counts on NA show the total surviving population (uninjured and sublethally injured) and counts on NA/SDS the uninjured population. The difference between them indicates the extension of the injury.
When the suspensions were rapidly chilled, *Pseudomonas* turned out to be more sensitive in terms of inactivation and injury. Its population reduced 0.4 log cycles, and *S. Enteritidis* PT4 and PT7 0.1 log. These results agree with the results in NB of the previous section. Presence of nisin during chilling, reduced further the population of salmonellas about 0.5 log and *Pseudomonas* about 1.2 logs, which is a little less compared to 0.8 and 2 logs respectively, that occurred with the presence of 500IU/ml nisin in NB.

The presence of EDTA in the chilled *Pseudomonas* suspension affected both viability and injury compared with the room temperature suspension, where EDTA caused only injury (sensitivity to NA containing SDS). Chilling and EDTA, like chilling and nisin, acted synergistically on the *Pseudomonas* population. While chilling reduced the population about 0.4 log cycles and EDTA at room temperature only 0.1 logs, these two treatments in combination resulted in a 1.7 log reduction. However, the same was not observed for the salmonellas.

When both nisin and EDTA were present in the chilled suspensions greater population reduction occurred for all microorganisms. A further 1.1 log reduction was observed for *Pseudomonas*, reaching 2.4 log cycles reduction, compared to 1.2 for chilling/nisin and 1.3 for chilling/EDTA. *Salmonella* Enteritidis PT4 population decreased 0.8 logs compared to 0.5 and 0.1 for chilling/nisin and chilling/EDTA respectively. The corresponding figures for *S. Enteritidis* PT7 were 0.5, 0.3 and 0.2 respectively.

**B 5.3.2 Decontamination of chicken skin**

The trials of similar combined treatments for the decontamination of chicken skins, were carried out using tap water and the results for *Salmonella* Enteritidis PT4 and *Pseudomonas aeruginosa* are illustrated in Figures B.5.4. and B.5.5, respectively. A previous experiment (results not shown) confirmed that there is no effect on the population due to any osmotic shock because of the immersion in tap water.

The results showed that the effect caused by the action of EDTA/nisin, chilling/nisin or chilling/EDTA/nisin was less pronounced than with treatments in PBS. This is clearer
Figure B.5.4. Effect of combinations of chilling, EDTA (20mM) and nisin (500 IU/ml) on decontamination of *Salmonella* Enteritidis PT4 on chicken skin. The error bars show the standard deviation of three replicates.
Figure B.5.5. Effect of combinations of chilling, EDTA (20mM) and nisin (500 IU/ml) on decontamination of Pseudomonas aeruginosa on chicken skin. The error bars show the standard deviation of three replicates.
for Pseudomonas where a satisfactory reduction of its population took place in PBS. The chilling/EDTA treatment was more effective than chilling/nisin, in contrast to the results in PBS. The combination of three factors reduced skin population of salmonellas by 0.6 log and Pseudomonas 1.1 log, in contrast to 0.8 and 2.8 respectively. In the case of Pseudomonas the 1.1 log difference is more due to the chilling effect alone rather than the effect of chilling/nisin and/or EDTA (Figure B.5.5).

B 5.4 DISCUSSION

The treatments in PBS at room temperature with nisin and with EDTA did not reduce the population of any of the bacteria tested. This is not unexpected, since nisin is not active against Gram-negative bacteria, and EDTA generally does not affect the viability of bacterial cells. Small reductions of course can sometimes occur (Delves-Broughton, 1993). However, EDTA affected the sensitivity of Pseudomonas aeruginosa to SDS, indicating that the permeability barrier of the outer membrane to such compounds was altered after the 10min exposure to 20mM EDTA. The same was not observed for the salmonellas. Hence, simultaneous presence of nisin and EDTA resulted in a decrease of Pseudomonas aeruginosa viability, but not of salmonellas, confirming that nisin could pass through only the damaged outer membrane barrier and inactivate the cell. Other researchers found not only Pseudomonas but also Salmonella species to be sensitive to the action of nisin with chelators, especially EDTA (Cutter and Siragusa 1995; Stevens et al., 1991). However, in the majority of these cases the conditions such as, buffers, exposure time, bacterial strains, nisin and EDTA concentration were not the same.

Both Salmonella Enteritidis PT4 and PT7 were not susceptible to the action of EDTA and EDTA/nisin, at least for the short period of 10 min. This indicates that the shorter chain length of the lipopolysaccharides of the outer membrane of PT7 strain does not facilitate EDTA access to the sites where the divalent Mg$^{2+}$ and Ca$^{2+}$ ions retain the lipopolysaccharide ‘tiles’ in order.

Rapid chilling, as was found in the previous section, sensitised the bacteria to nisin and the population reductions in PBS were quite similar to those observed in NB, showing that the two different treating menstrua did not affect the phenomenon significantly.
The presence of EDTA or both EDTA/nisin in the chilled *Pseudomonas* suspension acted synergistically, reducing the population far more than the individual factors. The same was not seen for salmonellas, where the effect seems to be more additive than synergistic. The effect of chilling on outer membrane structure and organisation seems to assist EDTA in disturbing further the permeability barrier, by chelating Mg$^{2+}$ and Ca$^{2+}$ ions causing more leakage of cellular materials, leading to cell death and facilitating nisin access to the cell membrane, enhancing the bacterial inactivation.

Different microorganisms exhibited different sensitivity to the chilling and/or EDTA and nisin. This variability is possibly due to differences in the outer membrane, cell membrane or other structural and compositional differences of the cell, as discussed extensively in section A.

Numerous attempts have been made to apply nisin in decontamination of surfaces and especially carcass surfaces. The combination of nisin with a chemical which will assist its access to the cytoplasmic membrane, like EDTA (Shefet *et al.*, 1995; Cutter and Siragusa 1995b) and trisodium phosphate (Carneiro de Melo *et al.*, 1997) has been investigated. In all cases, the population of the attached bacteria on the carcasses surface after the treatments, did not reduce or reduced less than with suspended bacteria in buffers. It is generally accepted that bacteria attached to surfaces are not readily accessible by various compounds, compared to suspended bacteria. Nisin also binds on food surfaces and it is not available for antibacterial action. It is also probable that a fraction of the EDTA chelates divalent cations from the carcass, hence the concentration that is available reduces.

The chilling shock presumably is not as severe as in buffers. Suspended bacteria at room temperature reduce their cell temperature extremely rapidly when they are added to a chilled buffer, compared to the attached bacteria on a surface. In the latter case, the bacteria will experience a slower decrease in temperature due to a thermal buffering effect as the chicken slowly cools.

From our investigation, chilling shock of carcasses and exposure in 20mM EDTA and 500IU/ml nisin for 10 min was not adequate for a satisfactory decontamination from
pathogenic and spoilage Gram-negative bacteria. The effect of all these factors together proved to be if not synergistic in all cases, at least additive. Since chilling is used in carcass handling, it could be used together with nisin and chelators treatment in formulations and exposure times that were found effective (Shefet et al., 1995), resulting to a more useful outcome, but this would require further refinement of the technique.
CHAPTER 6

CHARACTERISATION OF CELL WALL DAMAGE

AND ITS RELATION TO NISIN SENSITIVITY
CHAPTER 6

CHARACTERISATION OF CELL WALL DAMAGE AND ITS
RELATION TO NISIN SENSITIVITY

6.1 INTRODUCTION

It is known that the cell wall and the outer membrane of Gram negatives are susceptible
to damage by various physical or chemical treatments (Ray 1986). A variety of changes
in the structure and functions of the outer membrane can occur, including
morphological and structural changes, involving blebs and vesiculation, damage or
release of lipopolysaccharides (Hitchener and Egan 1977; Ray et al., 1976; Kempler and
Ray 1978; Katsui et al., 1982; Tsuchido et al., 1985). These changes alter the
permeability barrier, causing efflux of periplasmic enzymes, sensitivity to hydrophobic
compounds, dyes and surfactants (Strange and Postgate 1964; Ray et al., 1976; Kempler
and Ray 1978; Tsuchido et al., 1985; Tsuchido and Takano 1988), and changes of cell
surface hydrophobicity (Mackey 1983; Tsuchido et al., 1985).

Cell hydrophobicity is a property of the cell surface and is involved in interfacial
interactions of microbial cells with other microbial cells (flocculation), liquids, solids
(adsorption) and gases (flotation) (Mozes and Rouxhet, 1990). Since hydrophobicity is an
attribute of the surface and the cell surface is in direct contact with the environment, cell
surface hydrophobicity is involved in a lot of important phenomena. Aspects of
pathogenicity (Finlay and Falkow, 1989) and attachment to host tissue (Smyth et al.,
1978) are related to cell surface hydrophobicity, as are adhesion to various surfaces,
(Rosenberg and Kjelleberg, 1986) like attachment to food and food equipment surfaces
(Dickson and Koohmaroaia, 1989; Zottola 1994).

The term ‘hydrophobicity’ means literally ‘water aversion’. Hydrophobic effects have
been said to arise because ‘like prefers like’, suggesting that molecules or particles will
interact among themselves or with the solvent only if they possess similar structural and
chemical characteristic among them or with the solvent (Duncan-Hewitt, 1990).
Microbial cells possess polar molecular groups on their surfaces and therefore are expected to exhibit reduced overall surface hydrophobicity (Mozes and Rouxhet, 1990). This is generally true, but different cell species or strains exhibit different surface hydrophobicity, which is related to the outer surface structure and composition. Increased cell surface hydrophobicity due to reduction of O-polysaccharide (and core oligosaccharides) in the lipopolysaccharide of rough mutants of *Salmonella* has been reported (Magnusson and Johansson 1977; Hermansson *et al.*, 1982). In addition to LPS, other surface components, like proteins and fimbriae, have been implicated in governing hydrophobicity (Irvin 1990).

Various methods have been employed to measure cell surface hydrophobicity. The most commonly used methods rely on bacterial adhesion, such as Microbial Adhesion to Hydrocarbons (MATH), Hydrophobic Interaction Chromatography (HIC), Salt Aggregation Test (SAT) and adhesion to hydrophobic solid surfaces. Other methods used are contact angle measurement (CAM), two phase partitioning (TPP) and the binding of molecular probes (Rosenberg and Doyle, 1990).

Treatments that can harm the bacteria might affect the cell surface hydrophobicity, especially those causing structural or conformational damage of the bacterium, such as heating, freezing and drying. Increased hydrophobicity has been reported from heating (Mackey, 1983; Tsuchido *et al.*, 1985), freezing, drying, irradiating and EDTA treated bacterial cells (Mackey, 1983). In many cases, cells that have undergone such injury become sensitive to hydrophobic compounds (Mackey 1983; Tsuchido and Takano 1988). It is known that these cells become sensitive as a result of outer membrane permeability barrier damage (Vaara 1992). Nisin is a hydrophobic molecule, and also contains a high proportion of basic amino acids which gives nisin a net positive charge (Hurst, 1981). The chemical nature and charge of the bacterial surface, which is expressed by hydrophobicity, may play an important role in the nisin sensitivity of the injured Gram negatives. For example it would be easier for a hydrophobic biocide to attach onto a more than a less hydrophobic cell surface and subsequently exert its action.

Lipopolysaccharides, are the main structural units of the upper layer of the asymmetric bilayer of the outer membrane, with phospholipids occupying the lower layer. Adjacent
polyanionic LPS molecules are linked electrostatically by divalent cations (Mg$^{2+}$, Ca$^{2+}$) in the OM, to form a stable ‘tiled roof’ on the surface of the OM (Vaara, 1992). This structure, which is in a degree responsible for the permeability barrier of Gram negatives (Nikaido and Vaara, 1985) can be altered or damaged by different means. It has been reported that treatments that alter the permeability induce release of lipopolysaccharides (Hitchener and Egan, 1977; Tsuchido et al., 1985) into the medium. However, these reports used only EDTA and heat treatments. Despite the observation that other treatments like freezing damage the lipopolysaccharides (Ray et al., 1976; Kempler and Ray, 1978), and cold shock increase the OM permeability (Strange and Postgate, 1964), measurements of release of LPS have not been carried out.

This chapter reports a study of the damage to the outer membrane as measured by changes in cell surface hydrophobicity and release of lipopolysaccharide, determine the role of these two aspects, on the different sensitivity that the injured Gram negative cells exhibit to nisin.

6.2 MATERIALS AND METHODS

6.2.1 Organisms

Salmonella Enteritidis phage type 4 P167807, and phage type 7 P469815/0 and Pseudomonas aeruginosa USCC 2186 were used. Details about the microorganisms, maintenance and resuscitation are given in Chapter 2 (General Materials and Methods).

6.2.2 Chemicals

n-hexadecane, for the MATH method and sepharose CL-4B and octyl sepharose CL-4B for the HIC were supplied by Sigma (Sigma-Aldrich Ltd, Pole, Dorset, UK). The boron/trifluoride methanol complex 14% w/v for the methyl-esterification of fatty acids was purchased from Sigma (Sigma-Aldrich Ltd, Pole, Dorset, UK). Chloroform and hexane solvents were HPLC grade from Aldrich (Sigma-Aldrich Ltd, Pole, Dorset, UK).
Tridecanoic, pentadecanoic, heptadecanoic, nonadecanoic, β-hydroxy tetradecanoic and dodecanoic fatty acids were purchased from Sigma (Sigma-Aldrich Ltd, Pole, Dorset, UK). Nonadecanoic, β-hydroxy tetradecanoic and dodecanoic fatty acid methyl esters were also supplied by Sigma (Sigma-Aldrich Ltd, Pole, Dorset, UK).

6.2.3 Stress treatments

Heating, rapid chilling and freezing treatments were carried out according to the protocol described in Chapter 5.

To minimise the variances due to the different batches of grown suspensions, one culture of microorganism was grown for each series of replicates, centrifuged, washed and resuspended to give a concentrated suspension. Specific volumes were then added to the treating medium, and the property (hydrophobicity or lipopolysaccharides) was measured. In such case paired observations between the control and each of the treated samples can be carried out. Control samples of all kinds were analysed. All the trials were carried out at least in triplicate.

6.2.4 Determination of Cell Surface Hydrophobicity

The methods used were a modification of microbial adhesion to hydrocarbons (MATH), first reported by Rosenberg et al (1980), using hexadecane, and hydrophobic interaction chromatography (HIC) first applied to bacterial cells by Smyth et al (1978). Changes in hydrophobicity during the treatments were determined using the MATH test only. Salmonella Enteritidis PT4, PT7 and Pseudomonas aeruginosa only were tested. Samples of the temperature treated or untreated control suspensions in NB were used.

6.2.4.1 Microbial Adhesion to Hydrocarbons (MATH)

The adhesion coefficient of cells between n-hexadecane and aqueous phase was measured as an index of their relative surface hydrophobicities. MATH involves the
adhesion of cells to the n-hexadecane droplets, which decreases the cell concentration in
the aqueous phase. The higher the decrease the greater the hydrophobicity of the
microorganism. The method was conducted as follows:

Cell suspensions at stationary phase grown in NB at optimum temperatures, were
centrifuged at 1500xg for 15min, the pellets washed and re-suspended in Phosphate,
Magnesium, Urea (PUM) buffer (22.2g/l K₂HPO₄ 2H₂O, 7.26g/l KH₂PO₄, 1.8g/l urea,
0.2g/l MgSO₄ 7H₂O, pH 7.1). The absorbance and the counts of the resulting suspension
were taken at 400nm and as spread plates on NA respectively. A 2 ml sample of the
suspension was mixed with 0.5 ml n-hexadecane, left to pre-incubate at room
temperature for 10min before vortexing for 60s. The system was left at room
temperature for 30min. The aqueous phase removed, A₄₀₀ was measured and spread
plates were carried out. The % of hydrophobicity was estimated by using the formula:
(1- A₄₀₀/A₁)×100 % and (1- cfu₂/cfu₁)×100 % where 1 and 2 are the measurements taken
before and after the mixing with the hexadecane respectively.

6.2.4.2 Hydrophobic Interaction Chromatography (HIC)

HIC is a chromatographic procedure based on hydrophobic interactions between
nonpolar groups on a gel bed and a solute, e.g. protein. The proportion of the cells
retained by a hydrophobic gel gives an indication of their hydrophobicity, the greater
the retention the greater the hydrophobicity. The method was carried out as follows:

Cell suspensions were centrifuged at 1500xg for 15min and the pellets washed and re-
suspended in high ionic strength buffer (10mM potassium phosphate + 1 M (NH₄)₂SO₄)
to give a population of approximately 10¹⁰ cells/ml. Columns comprised short-ended
glass Pasteur pipettes plugged with glass wool and filled with octyl sepharose CL-4B
(Sigma) or sepharose CL-4B (Sigma) as control column, to a height of 2cm. Columns
were equilibrated with 2ml buffer before the addition of 100µl of test cell suspension.
Cells were eluted with 2ml of buffer. A₄₀₀ readings and spread plates of the initial cell
suspension and the column eluate were conducted and the percentage of cells retained
within the gel matrix and the percent eluted, calculated. The percentage of retained cells
in the octyl sepharose column give a relative measure of the hydrophobicity of the cell population. The use of sepharose CL-4B as control column demonstrates the extent of non-specific, non-hydrophobic interaction in the columns. The percentages of cells eluted from the gel matrices were used to calculate the hydrophobicity index (HI) of the cell population.

\[
HI = \frac{\% \text{ cells eluted from control column} - \% \text{ cells eluted from octyl sepharose column}}{\% \text{ cells eluted from control column}}
\]

(van der Mei et al., 1987)

6.2.5 Determination of lipopolysaccharides

The assay of lipopolysaccharides was conducted using the sensitive and versatile method of gas liquid chromatography (GLC) according to Lambert (1991). The method measures the amount of \(\beta\)-hydroxy fatty acids which are characteristic constituents of lipid A. The amount of \(\beta\)-hydroxy fatty acids that remained on the whole cells after the treatments was measured. The dry matter of the suspension used was also determined and the amount of \(\beta\)-hydroxy fatty acids were expressed per mg dry cell mass.

An alternative method is the thiobarbituric acid method, which measures another marker compound of the LPS, the 2-keto-3-deoxy-manno-octulosonic acid (KDO), described by Karkhanis et al., (1978). The disadvantage of that method is the interference from the cellular deoxyribonucleotides, given that the method measures 2-deoxy sugars.

6.2.5.1 Experimental procedure of the assay

Suspensions in 10ml NB of *Salmonella* Enteritidis PT4, PT7 and *Pseudomonas aeruginosa*, at a concentration of approximately \(10^9\) cfu/ml in NB were treated by heating, rapid chilling and freezing as described in Chapter 5. After the treatments, the cells centrifuged at 1500g for 15 min, washed in saline (0.85% NaCl) and resuspended
in 1 ml distilled water. One ml of the suspension in distilled water was used for the assay of lipopolysaccharides.

The assay was carried out in 15ml hydrolysis tubes fitted with Teflon lined screw caps (Fisher Scientific UK). To each series of hydrolysis tubes, 40 μl of a 0.5mg/ml standard solution of nonadecanoic acid in hexane to act as an internal standard was added. The internal standard must be a fatty acid not present in the cell tested. After trials with tridecanoic, pentadecanoic, heptadecanoic and nonadecanoic acids, it was found that nonadecanoic acid was a suitable internal standard for both *Salmonella* and *Pseudomonas*.

After the solvent was allowed to evaporate at room temperature, 1ml of the suspension in distilled water was added to each of the tubes. Then 4ml of 6M HCl was added, the caps were sealed and the tubes heated at 100°C for 4 h in a heating dry block (Technne DB3A, Scientific Laboratory Supplies Ltd, Nottingham UK). This treatment allowed the chemical release of β-hydroxy fatty acids from the lipid A, by hydrolysing the ester bond between them. The tubes were cooled down and 4ml of boron trifluoride/methanol complex 14% w/v was added, in order to convert the fatty acids to fatty acids methyl esters. They were heated for 10 min at 80°C in the same dry block.

The fatty acids methyl esters that formed were extracted in the hydrolysis tubes by shaking vigorously twice with 2 ml chloroform:hexane (1:4) mixture. The upper phase was placed in a quick-fit test tube and reduced to dryness on a rotary evaporator (Buchi, rotavapor-R, Switzerland). The residue in each flask was re-dissolved in 50 μl of hexane and transferred to small vials for storage at -20°C.

**6.2.5.2 GLC analysis of β-hydroxy fatty acids**

For the GLC analysis of the fatty acid methyl esters a Chrompack CP-SIL 88, WCOT fused silica 30m (length) x 0.25mm (internal diameter) column was used. The GC apparatus was a Varian 3400. The carrier gas was helium of 18 psi pressure. The split ratio was 1/30. The detector was a flame ionization detector (FID) utilising a mixture of hydrogen and air. Temperature programmed conditions were used. Initial temperature
135°C, hold for 10 min then increase with the rate of 1°C/min up to 150°C and then increase up to final temperature of 200°C with a rate of 5°C/min. The final duration of the run was 35 min. The sample size was 1 μl.

The characteristic β-hydroxy fatty acids for the Enterobacteriaceae and *Pseudomonas* are the β-hydroxy tetradecanoic and dodecanoic fatty acids respectively (Meadow 1975). Ready prepared β-hydroxy tetradecanoic and dodecanoic fatty acid methyl esters were used as reference compounds. For these reasons, solutions of 1mg/ml in hexane were prepared and run separately and also used to spike up the corresponding peaks of the sample in order for them to be identified.

A calibration graph was necessary for the qualitative determination of the β-hydroxy fatty acids. For this reason, β-hydroxy tetradecanoic and dodecanoic fatty acids were purchased and solutions 0.5mg/ml in hexane were prepared. Series of hydrolysis tubes with 40 μl of 0.5mg/ml solution of nonadecanoic acid and a range of volumes (0, 10, 20, 30, 40, 50, 60 μl) of β-hydroxy tetradecanoic and dodecanoic fatty acids 0.5mg/ml were prepared. The solvent was allowed to evaporate at room temperature and 1ml of distilled water was added. Then the procedure of the paragraph 6.2.5.1 was followed. From the peak areas, the β-hydroxy/nonadecanoic acid ratios were estimated and plotted against the μg of β-hydroxy fatty acids.

### 6.2.5.3 Dry weight determination

Watch glasses removed from the oven operating at 105°C and placed in a desiccator for 2 hours. Then, they were weighed in an analytical balance and placed back to the desiccator. One ml of the suspension transferred on the watch glasses and left for 48 hours at 105°C to dry. Then the watch glasses with the dried suspension were placed in the desiccator for 2 hours and then re-weighed.
6.3 RESULTS

6.3.1 Cell surface hydrophobicity

The hydrophobicities of the bacterial cell surfaces were evaluated by MATH and HIC method using both absorbance at 400nm and counts on NA plates. The results are shown in Figures 6.1 and 6.2, respectively. The MATH method showed that *Salmonella* Enteritidis PT7 together with *E. coli* were the most hydrophobic followed by *S. Enteritidis* PT4 together with *Ps. aeruginosa* and finally *Ps. fragi*, which found to be the most hydrophilic. The HIC method showed that *S. Enteritidis* PT7 was the most hydrophobic followed by *S. Enteritidis* PT4 and *Ps. fragi, Ps. aeruginosa* and finally *E.coli*. *S. Enteritidis* PT7 exhibited the most hydrophobic cell surface recorded with both methods. The HIC gave much higher values than MATH method except for *E.coli*, which was lower. Both methods show that *Salmonella* Enteritidis PT7 was the most hydrophobic strain.

From the order of hydrophobicities that the two methods gave, it is clear that the correlation between the two methods is poor. Indeed, in Figure 6.3a, the poor correlation of two methods for the microorganism tested can be seen. The correlation between the two methods becomes better when the measurements taken for *E. coli* were excluded (Figure 6.3b), but generally the two methods do not correlate.

Changes of the hydrophobic properties occurred during heating, rapid chilling freezing/thawing, were recorded by using the MATH test. Paired observation t-tests were conducted between the controls and the treated samples to evaluate the degree of the difference in hydrophobicity. For the strains tested, the hydrophobicity increased during heating (Figure 6.4 and Table 6.1) and freezing (Figure 6.5 and Table 6.2) and did not change significantly because of chilling (Figure 6.5 and Table 6.2).

The hydrophobicity at zero time differ significantly (p<0.05) from that at 5, 10 and 20 min of heating, showing that the increase took place during the first 5 min of the heating and then remained constant. Indeed, t-tests between the control and the heated samples showed that there was a significant difference (Table 6.1), in contrast to the values taken
Figure 6.1. Percentage Hydrophobicity determined by MATH and HIC procedure, using absorbance at 400nm. The error bars show the standard deviation of the mean taken by at least 3 replicates.

Figure 6.2. Percentage Hydrophobicity determined by MATH and HIC method, using counts on NA. The error bars show the standard deviation of the mean taken by at least 3 replicates.
Figure 6.3. Correlation of the MATH and HIC hydrophobicity methods. 
(a) Including all microorganisms. (b) excluding *Escherichia coli*. 
Figure 6.4. Hydrophobicities changes during heating in NB, of *Salmonella Enteritidis* PT4, PT7 and *Pseudomonas aeruginosa* as recorded with the MATH method. The error bars show the standard deviation of the mean taken by 3 replicates.

<table>
<thead>
<tr>
<th></th>
<th>% Hydrophobicity ± st. deviation</th>
<th>% Increase of Hydrophobicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. Enteritidis PT4</td>
<td>S. Enteritidis PT7</td>
</tr>
<tr>
<td>Control</td>
<td>18.1 ± 3.3</td>
<td>34.8 ± 3.5</td>
</tr>
<tr>
<td>Heating (5 min)</td>
<td>29.9 ± 3.8 (0.019)</td>
<td>46.0 ± 4.6 (0.006)</td>
</tr>
<tr>
<td></td>
<td>65.2</td>
<td>32.2</td>
</tr>
<tr>
<td>Heating (10 min)</td>
<td>32.0 ± 4.1 (0.014)</td>
<td>44.2 ± 4.1 (0.008)</td>
</tr>
<tr>
<td></td>
<td>76.8</td>
<td>27.0</td>
</tr>
<tr>
<td>Heating (20 min)</td>
<td>33.7 ± 4.4 (0.027)</td>
<td>46.5 ± 5.0 (0.013)</td>
</tr>
<tr>
<td></td>
<td>86.2</td>
<td>33.6</td>
</tr>
</tbody>
</table>

Table 6.1. Effect of heating on cell surface hydrophobicity (average ± st. deviation of three replicates), percentage of increase because of treatments, and the p value (in parenthesis) of paired t-tests, between the treated samples and the controls.
Figure 6.5. Effect of chilling and freezing on cell surface hydrophobicity of *Salmonella Enteritidis* PT4, PT7 and *Pseudomonas aeruginosa*, determined with MATH method, recording absorbance at 400nm. The error bars show the standard deviation of the mean taken by 3 replicates.

<table>
<thead>
<tr>
<th></th>
<th>% Hydrophobicity ± st.deviation</th>
<th>% Increase of Hydrophobicity</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. Enteritidis PT4</td>
<td>S. Enteritidis PT7</td>
<td>Ps. aeruginosa</td>
</tr>
<tr>
<td>Control</td>
<td>17.0 ± 3.9</td>
<td>30.8 ± 5.1</td>
<td>20.2 ± 2.1</td>
</tr>
<tr>
<td>Chilling</td>
<td>15.2 ± 4.1 (0.384)</td>
<td>30.2 ± 7.3 (0.750)</td>
<td>21.6 ± 1.8 (0.303)</td>
</tr>
<tr>
<td>Freezing</td>
<td>21.4 ± 2.6 (0.047)</td>
<td>34.4 ± 5.4 (0.003)</td>
<td>25.3 ± 4.1 (0.049)</td>
</tr>
</tbody>
</table>

Table 6.2. Effect of chilling and freezing on cell surface hydrophobicity (average ± st. deviation of three replicates), percentage of increase because of treatments, and the p value of paired t-tests (in parenthesis), between the treated samples and the controls.
for 5th, 10th and 20th minute of heating. Freezing and thawing caused increase of the cell surface hydrophobicity, but not chilling (Figure 6.5 and Table 6.2). It can be seen from Table 6.2 that the p values for freezing were less than 0.05, in contrast to chilling (p>0.05). The increase of hydrophobicity was greater for heating compared to freezing. For S. Enteritidis PT4, PT7 and Ps. aeruginosa the increase in hydrophobicity was 65, 32 and 57 % respectively for heating, and 26, 12 and 25 % respectively for freezing.

According to the sensitivity results of the stressed bacteria to nisin (chapter 5) and the hydrophobicity and hydrophobicity changes results of the current chapter, it can be concluded that there is no relation among the hydrophobicity and nisin sensitivity. Indeed, Salmonella Enteritidis PT7 was the most hydrophobic strain and its hydrophobicity increased to the highest levels during heating but it was not the most or the least sensitive strain. For the freezing treatment, S. Enteritidis PT7 was more sensitive than PT4 strain but not more sensitive than Ps. aeruginosa, despite the lower hydrophobicity of the latter. For chilling treatments similar conclusions can also be drawn.

6.3.2 Assay of lipopolysaccharides

The amount of LPS hydroxy fatty acid markers remained on the whole cell, before and after the treatments, was determined by using GLC. From the chromatograms of the three strains used, the two salmonellas gave identical ones. Salmonella and Pseudomonas chromatograms can be seen in Figure 6.6.

The samples were spiked up with tridecanoic, pentadecanoic, heptadecanoic and nonadecanoic acid methyl esters in order to deduce which was suitable as an internal standard (Figure 6.7). From Figure 6.6 and 6.7, it can be seen that indeed nonadecanoic acid was a suitable internal standard in both cases.

The salmonellas and Pseudomonas contain β-hydroxy tetradecanoic acid and β-hydroxy dodecanoic acid, respectively (Meadow 1975). In order to confirm that, and locate the peaks, samples from the bacteria were spiked with pure β-hydroxy fatty acid
Figure 6.6. Chromatograms of (a) *Salmonella* Enteritidis PT4 and PT7 and (b) *Pseudomonas aeruginosa*
Figure 6.7. Chromatograms of (a) *Salmonella* Enteritidis PT4 and PT7 and (b) *Pseudomonas aeruginosa*, samples with added tridecanoic, pentadecanoic, heptadecanoic and nonadecanoic acids.
methyl esters. These chromatograms are shown in Figure 6.8. It can be seen from
Figures 6.6 and 6.8 that the salmonellas do contain β-hydroxy tetradecanoic acid and
the Pseudomonas β-hydroxy dodecanoic acid only.

Standard mixture of nonadecanoic acid, β-hydroxy tetradecanoic acid and β-hydroxy
dodecanoic acid methyl esters chromatogram is shown in Figure 6.9. The relative
retention times of β-hydroxy tetradecanoic acid and β-hydroxy dodecanoic acid, which
were taken by dividing the retention time by the retention time of nonadecanoic acid
(internal standard), were found to be 1.18 and 0.80 min respectively. Typical
chromatograms of Salmonella and Pseudomonas with the addition of the internal
standard are shown in Figure 6.10. The relative retention times of β-hydroxy
tetradecanoic acid and β-hydroxy dodecanoic acid, were estimated to be 1.19, and 0.81
min, respectively, confirming the reproducibility of our method.

6.3.2.1 Calibration curve

The calibration curve is shown in Figure 6.11. From the chromatograms, the area ratios
of β-hydroxy fatty acids peaks to internal standard (nonadecanoic acid) were calculated.
Extrapolating to the horizontal axis, the μg of the β-hydroxy fatty acid remaining on the
whole cells of the suspension was estimated.

6.3.2.2 Release of lipopolysaccharides

Paired observation, one tail t-tests were conducted between the controls and the treated
samples to evaluate the degree of the difference (more specifically for the one tail test,
how lower the treated sample values were). For this purpose the p value required for the
treated value to be significantly lower compared to the control is presented. The μg of
β-hydroxy fatty acid /mg dry mass of the suspension used ± standard deviation is shown
in Table 6.3. The percentage release of lipopolysaccharides is also shown.
Figure 6.8. Chromatograms of (a) *Salmonella* Enteritidis PT4 and PT7 and (b) *Pseudomonas aeruginosa*, spiked up with pure β-hydroxy dodecanoic and β-hydroxy tetradecanoic acid methyl esters.
Figure 6.9. Chromatogram of standard mixture of nonadecanoic, β-hydroxy tetradecanoic and β-hydroxy dodecanoic acid methyl esters.
Figure 6.10. Chromatograms of (a) *Salmonella Enteritidis* PT4 and PT7 and (b) *Pseudomonas aeruginosa*, samples with added internal standard (nonadecanoic acid).
Figure 6.10. Calibration curve of the area ratios between the β-hydroxy fatty acids and internal standard (nonadecanoic) acid, against the amounts of β-hydroxy fatty acids in micrograms (μg).
Table 6.3 The µg of β hydroxy fatty acid per mg dry matter ± st. deviation of 3 replicates are shown in the first line. In the second line the p values, where the values differ compared to the control, according to the paired t test is shown, and the last line is the percentage release of lipopolysaccharides.

<table>
<thead>
<tr>
<th>Condition</th>
<th>S. Enteritidis PT4</th>
<th>S. Enteritidis PT7</th>
<th>Ps. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.8 ± 1.1</td>
<td>5.1 ± 1.2</td>
<td>4.5 ± 0.4</td>
</tr>
<tr>
<td>Heating</td>
<td>3.4 ± 0.6</td>
<td>3.0 ± 0.6</td>
<td>4.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>0.011</td>
<td>0.048</td>
<td>0.187</td>
</tr>
<tr>
<td></td>
<td>40.9</td>
<td>42.3</td>
<td>*11.8</td>
</tr>
<tr>
<td>Chilling</td>
<td>4.9 ± 1.7</td>
<td>4.0 ± 1.0</td>
<td>3.9 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>0.189</td>
<td>0.071</td>
<td>0.316</td>
</tr>
<tr>
<td></td>
<td>*14.0</td>
<td>*21.4</td>
<td>*13.1</td>
</tr>
<tr>
<td>Freezing</td>
<td>3.3 ± 0.8</td>
<td>2.8 ± 0.2</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>0.019</td>
<td>0.035</td>
<td>0.028</td>
</tr>
<tr>
<td></td>
<td>43.1</td>
<td>46.1</td>
<td>34.0</td>
</tr>
</tbody>
</table>

* This difference is not significant
Comparing the amount of β-hydroxy fatty acids per mg dry cell matter of *S. Enteritidis* PT4 and PT7, which found to be 5.8μg and 5.1μg respectively (Table 6.3), it can be concluded that they do not differ significantly (*p*=0.46). This indicates that the PT7 rough strain contains equal amount of LPS compared to PT4 smooth strain, assuming of course that the only difference of these two strains is the length of the O-chain and there are no differences in the structure and composition of lipid A moiety. The amount of LPS is constant, regardless of the length of saccharide chain, confirming Smit *et al.*, (1975) findings, as PT7 is a strain with shorter LPS chains (Chart *et al.*, 1989), compared to PT4 one, and is not a deep rough strain, which usually incorporate 20-30% more LPS (Nikaido and Vaara 1985).

Heating caused release of LPS. However, only salmonelllas were found to suffer significant release of their lipopolysaccharides after ten minutes of heating at 55°C (Table 6.3). More specifically 41 and 42% of the total lipopolysaccharides for *Salmonella Enteritidis* PT4 and PT7 respectively, were released, but no significant release was detected for *Pseudomonas aeruginosa* (Table 6.3). *Pseudomonas aeruginosa* was the most resistant to the presence of nisin during heating and *S. Enteritidis* PT4 the most sensitive, according to the results of Chapter 5. The results show positive relation between, damage of the OM by release of lipopolysaccharides and ability of nisin to inactivate heat injured Gram negatives by passing the OM barrier.

Chilling did not cause significant release of LPS from any of the tested microorganisms (Table 6.3). However, the stressed cells, especially of *Pseudomonas aeruginosa*, found to be sensitive in nisin (Chapter 5).

Freezing-thawing stressed cells of *S. Enteritidis* PT4, PT7 caused loss of LPS to a similar extent (Table 6.3) and *Pseudomonas aeruginosa* a little less. The release was found to be 43, 46 and 34%, for *S. Enteritidis* PT4, PT7 and *Pseudomonas aeruginosa* respectively. *Pseudomonas aeruginosa* freeze-stressed cells were shown great reduction in viability in presence of nisin, compared to *S. Enteritidis* PT4 and PT7 (chapter 5), without having higher degree of OM damage in terms of LPS release.
6.4 DISCUSSION

The temperature stress applied to the microbial suspensions of *Salmonella* Enteritidis PT4, PT7 and *Pseudomonas aeruginosa* rendered them sensitive to compounds that they are usually resistant to, such as SDS or nisin (Chapter 5). It is accepted that the sensitivity that they exhibit to such compounds reflects changes of permeability properties, hence damage or alterations of OM structure. This damage may also be indicated by changes in cell hydrophobicity or release of lipopolysaccharides.

The cell surface hydrophobicity of the microorganisms was tested using the MATH and HIC method. HIC gave higher values than MATH methods with the exception of *E. coli*. The correlation of the two methods was very poor. Excluding the *E. coli* values, the correlation became better but still not satisfactory. Both these methods claim to measure similar properties of the cell surface as they both based on hydrophobic adhesion. Whilst some other workers have reported a good correlation between these two methods (Darnell *et al.*, 1987), it is not uncommon for others to report a poor correlation (van der Mei *et al.*, 1987). Generally the hydrophobic properties rely on the LPS, but other surface components, like proteins and fimbriae, have been implicated in governing hydrophobicity (Irvin, 1990). It seems that differences on the surfaces of the different microorganisms used reflect different response to these methods (e.g. *E. coli*).

The increased hydrophobicity found for *Salmonella* Enteritidis PT7 strain compared to PT4, confirm the results of other researchers, who reported increased cell surface hydrophobicity due to reduction of O-polysaccharide (and core oligosaccharides) in the lipopolysaccharide of rough mutants of *Salmonella* (Magnusson and Johansson 1977; Hermansson *et al.*, 1982).

Heating and freezing increased the hydrophobicity in contrast to chilling which did not cause any change. Mackey, (1983) and Tsuchido *et al.*, (1985) reported increase of hydrophobity of *E. coli* due to heating. Mackey (1983) also found a great increase of *E. coli* cell surface hydrophobicity due to freezing-thawing. Both of them found the treated cells to be sensitive to hydrophobic compounds. In our study the different hydrophobicities found, and their increase because of the treatments, did not correlate to
the nisin sensitivity of the injured Gram-negative (according to the results of Chapter 5). However, Kobayashi et al., (1991), correlated the higher sensitivity of Bacteroides fragilis to hydrophobic antimicrobials (lincomycin and clindamycin), compared to Salmonella typhimurium and Pseudomonas aeruginosa, to the higher hydrophobicity of the former. The cell surface of resistant cells of Listeria monocytogenes was less hydrophobic and bound less nisin compared to the sensitive cells (Davies and Adams 1994; Ming and Daeschel 1995; Davies et al., 1996). However, taking into account the observation of Chapter 5 and especially that chilled bacteria were sensitive to nisin regardless that chilling did not cause change in hydrophobicity, it can be concluded, that hydrophobicity and its changes is not a prime factor that affects the sensitisation of Gram-negatives to nisin.

The fact that thermal stress damages the OM by releasing lipopolysaccharides was confirmed in our study. Results of other researchers have reported the release of that main component of the OM (Hitchener and Egan 1977, Tsuchido et al., 1985), complexed with protein and lipids as vesicles (Katsui et al., 1982). Freezing-thawing stress was also found to cause release of LPS molecules. Previous researchers have reported that both conformational alteration and structural damage of LPS occurred because of freezing (Ray et al., 1976; Kempler and Ray 1978), indicating damage in the heptose region with probable loss of heptose, but loss of LPS has not been reported. In contrast to heating and freezing, chilling did not cause any release of lipopolysaccharides. Nevertheless, chilled cells showed sensitivity to nisin. Other researchers have confirmed that chilled stressed Gram-negative cells become permeable to compounds that the healthy do not (MacLeod and Calcott, 1976).

Greater release reflected a greater sensitivity of the thermally injured cells to nisin. This shows that the structural disorganisation of the adjacent polyanionic LPS molecules with their concomitant release to the environment is a main cause for the sensitisation of thermally injured cells to nisin. However, the extention of the release due to freezing was not correlated with the degree of sensitisation of the Gram-negatives tested (S. Enteritidis PT4, PT7 and Ps. aeruginosa) to nisin. Chilled bacteria were sensitive to nisin regardless that chilling did not cause significant release of LPS. Therefore, the release of LPS, which is known to cause great OM permeability damage, is not the exclusive factor which is required to render the cells susceptible to nisin. It seems that
alteration of outer membrane permeability to nisin occurs by means which are not related to the structural damage. Conformational alterations, which may occur because of chilling, and release of LPS by the other two treatments, lead to the increase of the permeability.

Nevertheless, chilled cells were not sensitive to SDS but were to nisin and freeze-thawed cells were less sensitive to SDS than nisin (Chapter 5), despite the lower molecular weight of the former. This might be due to experimental artifact or to a 'self-promoted' uptake of nisin by the injured cells, as discussed in Chapter 5. Healthy cells do not exhibit such property (Vaara 1992; present thesis appendix 2) but cells, which have suffered conformational alteration of outer membrane, might do. However, this requires further investigation.

Cells injured by heating, freezing and chilling, showed transient susceptibility to nisin (Chapter 5), which indicates reorganisation or a kind of recovery of the OM after the treatments. According to Nikaido’s model (Nikaido and Vaara 1985), release of LPS from the outer asymmetric bilayer of Gram-negatives by the action of EDTA, results in the occupation of these sites by phospholipids which fill the void left creating a hydrophobic path, making the cells sensitive to hydrophobic compounds. According to that theory the heated and frozen cells, which undergo release of LPS should not exhibit transient susceptibility to nisin. Addition of nisin after the treatments should have caused inactivation of the cells, since nisin could have gained access through the hydrophobic path. It is apparent that there must be another way of handling the void space left after the release of LPS, or the hydrophobic paths are not big enough to facilitate the passage of a quite big molecule like nisin. Biosynthetic repair might occur but seems unlikely to have a great contribution in recovery, since nisin was added straight after the treatments. These aspects require further investigation. Whatever really happens, reversible permeability damage by physical treatments has been reported, for heating, freezing, chilling (present thesis Chapter 5) and high pressure (Hauben et al., 1996). In all these cases the nature of recovery has not been completely elucidated. Reorganisation of the existing molecules seems to be able to restore barrier functions, without synthesis of LPS, especially in cases where no or little loss of LPS occurs. (Ray 1993).
A relationship between hydrophobicity changes and release and/or damage of LPS is apparent. Cell wall damage as release of lipopolysaccharides was recorded for heating and freezing, but not for chilling. Heating and freezing also increased the cell surface hydrophobicity while chilling did not. It is apparent that LPS has an important influence on cell surface hydrophobicity. Increased cell surface hydrophobicity of rough mutants due to reduction of O-polysaccharide (and core oligosaccharides) in the lipopolysaccharide of *Salmonella*, have been reported (Magnusson and Johansson 1977; Hermansson *et al.*, 1982). Hence, the damage and release of lipopolysaccharides render the outer surface of the cells more hydrophobic, since there are not enough hydrophilic sugar chains to protrude, and because of the occupation of the empty sites by phospholipids, after the removal of LPS, according to Nikaido’s model (Nikaido and Vaara 1985).
CHAPTER 7

GENERAL DISCUSSION, CONCLUSIONS

AND

PROPOSALS FOR FUTURE WORK
CHAPTER 7

GENERAL DISCUSSION, CONCLUSIONS AND PROPOSALS FOR FUTURE WORK

Current interest in food science and technology is towards the production of mild treated foods free, as far as possible, from chemical preservatives. To reduce physical processing (heating, freezing etc), without use of chemical additives, it would be helpful to employ natural preservatives, to help maintain safety and reasonable shelf life. ‘Hurdle’ technology (Leistner and Gorris 1995) is an approach to this concept. The synergistic or combined action of nisin with OM permeabilising agents or physical treatments against Gram-negative bacteria could be part of a hurdle or multiple barrier approach to maintain food safety. The presence of nisin in combination with a mild preservation technique and/or chelators, other bacteriocins, lysozyme or other natural antimicrobials, multi-targeting onto the microbial cell could achieve mild but reliable preservation. Since nisin is a well-established, approved natural preservative with very appreciable effect against Gram-positives, this encourages the attempts to expand its action against Gram negatives.

Very recent publication on that subject studied, the presence of lipids, proteins, divalent cations, pH, NaCl, EDTA and propyl-paraben on nisin effectiveness against a range of Gram-positive and negative bacteria (Ganzle et al., 1999). The synergistic effect of nisin with chelators is a well-studied combination (Stevens et al. (1991; 1992a; Delves-Broughton 1993; Cutter and Siragusa 1995a). Chelators damage and increase the permeability of the OM by removing the divalent cations responsible for LPS stabilisation (Hancock 1984; Vaara 1992), hence nisin is able to reach its action site, the cytoplasmic membrane. The application of nisin and chelators might be promising but the in situ production of nisin and chelators seems problematic. Lactic acid bacteria, which produce nisin, also drop the pH of the surrounding environment. Despite the fact the low pH is favourable for nisin stability and action its inhibits the chelating action of EDTA and pyrophosphates tested.
Furthermore, the developed acidity (lactic acid) did not cause any synergistic effect with nisin.

The application of nisin in combination with physical processes like heating, chilling, freezing, studied in this thesis, or with novel preservation techniques like high-pressure and electroporation, studied by other researchers (Kalchayanand et al., 1994; Hauben et al., 1996), looks promising since these treatments cause structural injury to the OM of Gram-negative bacteria. All the treatments are able to cause synergistic effect with nisin giving the prospect of milder process. Reduction of D values (Boziaris et al., 1998) and pressure-time combinations (Kalchayanand et al., 1994) are feasible. Although the food environment opposes the effective action of nisin, applications still look potent. Food surfaces and the fatty components bind nisin (Daeschel 1990), and a high pH inactivates it (Hurst 1981), as probably happened in the experiments reported here with chicken surfaces, liquid whole egg and egg white respectively. Nevertheless applications in minimal fat foods with acidic pH and addition of small amounts of chelators or other bacteriocins that act synergistically, like pediocin, to nisin action, seem more feasible. A subject for future research could be the synergistic effect of other potentially useful bacteriocins and natural antimicrobials with heating, freezing, freeze-drying, chilling and acidification, as well as emerging and novel food preservation methods, such as, high-pressure, electroporation, and sonication.

Another aspect of great practical interest for the applications is the fact that the synergistic effect of nisin with most of the physical treatments is transient. Addition of nisin after heating, chilling, freezing (present thesis) and high-pressure caused no effect (Hauben et al., 1996). Even the sequential application of nisin to EDTA-treated cells gave less pronounced results compared to its simultaneous application (Stevens et al. 1991). The cell's permeability barrier recovers rapidly after treatments so survivors are no longer sensitive to nisin, although recovery is not sufficient to exclude smaller molecules such as NPN and SDS (present thesis, Hauben et al., 1996). The results further emphasise the value of simultaneous application of inimical treatments rather than their sequential application.

The majority if not all of the synergistic or combined effects with nisin, require permeabilisation of the Gram-negative OM. Nisin can only pass through damaged OM
membranes and reach the cytoplasmic membrane. LPS seems to be the most important molecule, which contributes to the OM permeability barrier. Mutants with defective LPS or spheroplasts from Gram-negative bacteria are sensitive to nisin (Stevens et al., 1992b; Scheved et al., 1994). Release of LPS is a common feature in permeabilisation of OM by chelators, heating and freezing (present thesis, Tsuchido et al., 1985; Leive 1974) which reflects and increases in cell surface hydrophobicity (present thesis Tsuchido et al., 1985). To study the effect of LPS chain length, Salmonella Enteritidis PT4 and PT7 strains were used in our study. PT4 is the wild type with PT7 possessing shorter LPS chains (Chart et al., 1989). The use of a deep rough strain was not of interest, because these cells are sensitive to such compounds due to the phospholipid patches on their outer membrane (Nikaido and Vaara 1985). The shorter LPS chain length did not increase the injury and sensitisation to nisin in the case of heating and EDTA treatment, but influenced the sensitisation of freeze-injured cells. If the main factor in these cases is the release of LPS, heating and EDTA were not obstructed or facilitated by the longer or shorter chain length and produced similar injury and sensitisation to nisin. In the case of freezing though, the strain with the shorter LPS chain was more sensitive not only to the presence of nisin but also the presence of SDS in the treating medium. In that case it seems that the protruding chains from the cell surface protect the cells from the mechanical pressure of the ice crystals.

Despite the fact that the majority of the treatments responsible for the sensitisation of Gram-negatives to nisin and other compounds cause loss of LPS, the same was not observed in the case of chilling-sensitised cells. Likely LPS are very critical but possibly not the exclusive molecules contributing to OM barrier integrity. A great percentage of the OM surface area of Gram-negatives is covered by proteins (Nikaido 1996). Yeast cells, which are known to be resistant to nisin, contain proteins in their cell wall, which prevent them from being sensitive to nisin (Dielbandhoesing et al., 1998). Further than the LPS which have been studied quite extensively (present thesis, Hitchener and Egan 1977; Ray et al., 1976; Kempler and Ray 1978; Tsuchido et al., 1985), it would be very interesting to look for any damage or loss of OM proteins in stressed Gram-negative bacteria. The presence and the interactions between proteins with other proteins, lipoproteins and LPS of the OM might be important. The sensitisation of chilling stressed cells to nisin might also be due to conformational
alterations rather than structural damage. Conformational alterations likely contribute to the sensitivity of cells treated by other means as well (Ray et al., 1976; Kempler and Ray 1978; Ray 1993). The rapid chilling, that was used in our study, possibly caused rapid solidification of either phospholipids in the inner layer or lipids chemically bonded to lipid A of LPS, and a weakening of all kinds of interaction in OM. This could cause cracks and a loosened association of OM constituents.

The cells recovered very fast when the stress (heating, chilling, and freezing) was removed. This indicates structural reorganisation rather than de novo synthesis and biosynthetic repair. In the case of chilling the reorganisation seems very likely but heating and freezing caused loss of LPS and has not been elucidated, how the rest of the molecules can give surface not permeable to nisin. However, smaller molecules were still able to penetrate the OM. Structural reorganisation of the existing molecules rather than synthesis is possible (Ray 1993), but the whole subject is a chance for future investigation.

The permeability barrier of the OM of Gram-negative bacteria against a lot of hydrophobic and amphiphilic compounds is well established but might not be the only factor that contributes to resistance of Gram-negatives to such compounds including nisin. The latest views discuss the existence of energised efflux pumps, which contribute together with OM barrier to the resistance of Gram-negatives to antibiotics (Nikaido 1996). The existence of efflux pumps for a variety of antibiotics, used in antimicrobial chemotherapy, has now being established (Hancock 1997; Nikaido 1998). It would be very interesting to examine the possibility of occurrence of such efflux pumps for molecules like nisin.

Nisin against Gram-negatives is a subject that requires further investigation, especially the integrity and restoration of OM that occurs after physical processes. From technological point of view, food properties that inhibit its action should be further studied to figure out the food environments where nisin can successfully be applied, for effective action not only against Gram-positives but also Gram-negatives.
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APPENDICES
APPENDIX 1

In this appendix, the growth curves of Salmonella Enteritidis PT4, P167807, PT7, Escherichia coli ATCC 25922, Pseudomonas aeruginosa USCC 2186, and Pseudomonas fragi ATCC 4973 used in the experiments are shown.

Growths were measured in NB incubated statically at 37°C (25°C for Pseudomonas fragi). The populations were monitored by counting on NA, using the spread plate technique.

For all nisin experiments, stationary phase cells were used. The Enterobacteriaceae were treated after 18-20 h and the pseudomonads after 24 h incubation.
APPENDIX 2

Appendix 2 shows the effect of nisin on uninjured Gram negatives. The microorganisms used were *Salmonella Enteritidis* PT4 and PT7, and *Pseudomonas aeruginosa*.

Appendix 2a The effect of nisin on growth of uninjured Gram negatives was carried out by taking absorbance measurements at 600 nm. NB was inoculated with the microorganisms and incubated at 37°C statically. When the population reached the detection level of the spectrophotometer (about 0.05 at 600 nm), nisin added in different concentrations. The results are shown in the graphs, below.
As it can be seen from the Figures shown above, high concentrations of nisin (≥1500 IU/ml) reduce the growth rate of Gram negatives, showing an inhibitory action. The inhibitory action is more pronounced for *P. aeruginosa*.
Appendix 2b The effect of nisin on injury and inactivation of uninjured Gram negatives was carried out in PBS incubating statically at 37°C for 5 days. PBS was inoculated with the microorganisms and different concentrations of nisin were added. The population was monitored by plating on NA and NA supplemented with 0.15% SDS.

Effect of nisin (2500 IU/ml) on injury and inactivation of healthy cells of Salmonella Enteritidis PT4 in PBS at 37°C. Almost identical results were taken for the other two microorganisms, showing that nisin was unable to cause either reduction of Gram-negatives population or injury. The counts taken in presence of nisin were not different from those taken for the control and the counts on NA/SDS were not different from those on NA respectively.