APPLICATION OF LYSOZYME AND NISIN TO CONTROL BACTERIAL GROWTH ON CURED MEAT PRODUCTS

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Submitted to the Faculty

of Graduate Studies

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By

Alexander Ogilvie Gill

In Partial Fulfillment of the

Requirements for the Degree

of

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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FOREWORD

This thesis is composed of four papers prepared for journal publication with the addition of a literature review (Chapter 2), and consolidated introduction (Chapter 1), conclusion (Chapter 7) and references. The Chapters 3 to 6 are presented as originally submitted for publication, with minor changes to format for the purposes of standardizing presentation of this thesis.

Chapter 3, "*In vitro* evaluation of antimicrobial combination treatments using response surface analysis of checker board experiments", was originally prepared as a report for Canadian Inovatech Ltd. This paper has not been submitted for publication as the experiments described were superseded by the work described in Chapter 4. Chapter 3 was included in the thesis so that a comparison may be made with the results described in Chapter 4.

Chapter 4, "Interactive inhibition of meat spoilage and pathogenic bacteria by lysozyme, nisin and EDTA in the presence of nitrite and sodium chloride at 24 °C", was submitted to the journal Lebebsmittel-Wissenschaft und Technologie, on November 18 1999.

Chapter 5, "Inhibition of bacterial growth on ham and bologna by lysozyme, nisin and EDTA", was accepted for publication in the journal Food Research International, on November 1 1999.

Chapter 6, "Surface application of lysozyme, nisin and EDTA to inhibit spoilage and

pathogenic bacteria on ham and bologna", was submitted for publication in the Journal of Food Protection, November 29 1999.

ABSTRACT

Chemical preservatives are increasingly unacceptable to consumers, while demand is increasing for minimally processed and convenient food products. Response to this situation requires the development of novel preservation strategies. Potential alternatives to traditional chemical preservatives are the enzymes lysozyme and nisin, which can be perceived by consumers as natural, due to their biological origin. Reports published by other authors have indicated that interaction between lysozyme or nisin with chelators may result in an increased antimicrobial effect against Gram positive and Gram negative organisms.

Experiments were conducted in nutrient broth using organisms of concern for safety or spoilage reasons in cured meat products. The individual antimicrobial effect of lysozyme, nisin, ethylene diamine tetraacetate (EDTA), tripolyphosphate and diacetyl was determined. A response surface analysis of fractional inhibitory concentration data was conducted to determine what, if any, interactions occurred between lysozyme and the other agents, and to determine if lysozyme potentiated the action of any of the other antimicrobials.

Potentiation of activity by lysozyme was observed in two cases: lysozyme with nisin against *Brochothrix thermosphacta* and lysozyme with EDTA and diacetyl against *Staphylococcus aureus*. The trials conducted did not indicate the potentiation of effect in any of the other combinations tested.

A two level, five factor, full factorial experimental design was also used to screen for interactions between the antimicrobials, lysozyme, nisin, EDTA, NaCl and $NaNO_2$. NaCl and

$NaNO_2$ were included so as to determine if their presence in cured meat products could have an interactive effect with the other antimicrobials. Fourteen organisms of concern in the spoilage or safety of cured meat products were grown in broth media. The absorbance at 450 nm relative to inoculated controls was determined after 72 h.

Agents alone reducing growth were: lysozyme against *Brochothrix thermosphacta, Pediococcus acidilactici, Enterococcus faecalis* and *Weissella viridescens*; nisin against all Gram positive organisms tested, including *Lactobacillus sakei* and *Pediococcus pentosaceus*; EDTA against all organisms tested; NaCl or nitrite against *Escherichia coli* O157:H7, *Salmonella typhimurium, Serratia grimesii*, and *Shewanella putrefaciens*; nitrite against *Lactobacillus curvatus, Leuconostoc mesenteroides, Listeria monocytogenes* and *Staphylococcus aureus.*

Interactions were observed between: lysozyme and EDTA (*En. faecalis* and *W. viridescens*); nisin and EDTA (all Gram positive organisms); EDTA and NaCl (*E. coli, S. typhimurium, Ser. grimesii*); EDTA and nitrite (*E. coli, Lb. curvatus, Lc. mesenteroides, L. monocytogenes, S. typhimurium*); nisin and nitrite (*Lc. mesenteroides, L. monocytogenes*); NaCl and nitrite (*S. typhimurium, Sh. putrefaciens*). All interactions resulted in reduced growth.

Application of a lysozyme, nisin and EDTA combination treatment to cured meat products was then investigated. Ham and bologna sausages were prepared with or without addition of 500 mg/kg lysozyme:nisin::1:3, and 500 mg/kg EDTA. Sausages were inoculated

with one of: *Brochothrix thermosphacta*, *Escherichia coli* O157:H7, *Lactobacillus sakei*, *Lactobacillus curvatus*, *Leuconostoc mesenteroides*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Serratia grimesii* or *Shewanella putrefaciens*, vacuum packed and stored for 4 weeks at 8 °C. Plate counts were made on selective and nonselective media.

Inhibitor treatment reduced initial populations of *B. thermosphacta* and *Lc. mesenteroides* on both meats. Treatment of ham and bologna prevented growth of *B. thermosphacta*, to week 4. Treatment reduced growth of *Lb. curvatus* on ham and bologna, to week 3. Treatment of bologna reduced growth of *Lc. mesenteroides* and *L. monocytogenes* for 2 weeks. Treatment of ham reduced growth of *E. coli* O157:H7 for 4 weeks. On treated ham the growth of *S. typhimurium* increased from week 3. No difference was observed between control and treatment samples with other organisms.

Since the growth of bacterial pathogens and spoilage organisms is localized to the surface of cured meat products it was theorized that immobilization of the antimicrobials in a surface coating may result in improved antimicrobial activity. Cooked ham and bologna sausage was prepared and received one of three treatments: no coating (control); coating with a 7% (w/v) gelatin gel (gel-control), or coating with a 7% gelatin gel containing 25.5 g/liter lysozyme:nisin::1:3 plus 25.5 g/litre EDTA (gel-treated). The samples were then inoculated with one of six test organisms: *Brochothrix thermosphacta; Escherichia coli* O157:H7; *Lactobacillus sakei; Leuconostoc mesenteroides; Listeria monocytogenes*, or *Salmonella typhimurium*. Inoculated samples were vacuum packed and stored at 8 °C for 4 weeks.

The antimicrobial gel treatment had an immediate bactericidal effect up to 4 log CFU/cm^2 on the four Gram positive organisms tested (*B. thermosphacta*, *Lb. sakei*, *Lc. mesenteroides* and *L. monocytogenes*) and inhibited the growth of these organisms over the 4 weeks of storage.

The antimicrobial gel treatment also had a bactericidal effect on the growth of *S. typhimurium* over the period of storage.

The numbers of *E. coli* O157:H7 on ham were reduced by 2 log CFU/cm^2 following treatment with both antimicrobial-containing and non-antimicrobial gels over the 4 week storage period. No effect was observed upon the growth of *E. coli* O157:H7 on bologna.

These experiments indicate that a lysozyme, nisin and EDTA combination treatment may be highly effective in controlling the growth of Gram positive bacteria on cured meat products. However, the treatment was of limited use against the Gram negative bacteria studied. Future development of this research should be focused on the development of packaging materials to serve as a delivery system for the antimicrobials and the investigation of applications to fresh meat products.

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1. Introduction

Consumers increasingly demand foods that are microbiologically safe, minimally processed and have lower levels of chemical preservatives, yet at the same time are convenient, with long shelf lives. Currently there is much research interest in developing novel food preservation systems in an attempt to resolve the conflict between these goals. One approach to this problem is the development of new food preservative systems that are more acceptable to consumers than so called "chemical preservatives."

Lysozyme and nisin are two antimicrobial peptides which may be suitable for the development of novel preservative systems. Both lysozyme and nisin may be perceived by consumers as "natural antimicrobials" as they are produced from organisms. Lysozyme is extracted commercially from chicken eggs and nisin is produced by *Lactococcus lactis* during fermentation of milk. Both peptides possess antimicrobial activities that are directed at bacterial cell structures that are not present in human cell.

The main obstacle to the application of these antimicrobial peptides is their restricted range of activity. Both peptides are effective against a range of Gram positive bacteria, but are ineffective against Gram negative bacteria. This is particularly of concern in the control of the Gram negative bacterial pathogens, *Brucella*, *Camplylobacter*, *Escherichia coli*, *Salmonella*, *Shigella*, *Vibrio* and *Yersinia*.

As a consequence of these limitations, two lines of research into the application of

lysozyme and nisin have developed. Firstly, sensitive organisms and their growth conditions are identified with a view to the use of lysozyme and nisin in specialized applications where their presence may be used to control a specific unwanted organism. Secondly, attempts have been made to increase the susceptibility of Gram negative organisms to these agents by using them in combination with other antimicrobials, or in the case of lysozyme by modification of the molecule to alter its function and properties.

The research program detailed in this thesis was undertaken with the objective of determining the effectiveness of a lysozyme and nisin-based antimicrobial system against a wide range of organisms and to investigate application of such a system to cured meat products. Several papers have been published previously on the topic of antimicrobial interactions, particularly on the use of nisin with ethylene diamine tetraacetate (EDTA) to target Gram negative organisms, but these experiments were deeply flawed, with studies conducted only against cells under starvation or other growth restrictive conditions. Thus it was necessary to perform an investigation of antimicrobial interactions under conditions of growth that are more representative of food products. The work described in this thesis on the application of a lysozyme, nisin and EDTA antimicrobial system to cured meat products is, to the best of my knowledge, the first work to be published on application of lysozyme or nisin as a preservative of these types of product.

2. Literature Review

2.1. Lysozyme

Lysozyme is the name for a class of enzymes which hydrolyze the β(1-4) glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine of peptidoglycan which compose the cell wall of Gram positive and Gram negative bacteria (Fig. 2.1). The discovery of lysozyme is attributed to Alexander Flemming, who accidentally observed, in 1922, that nasal mucous could dissolve bacterial colonies on an agar plate (Proctor and Cunningham, 1998). Since then lysozymes have been isolated from a wide range of sources including; mammalian tears, mucous, milk, tissues and serum, avian eggs, and some vegetables particularly of the Brassicaceae family (Proctor and Cunningham, 1998).

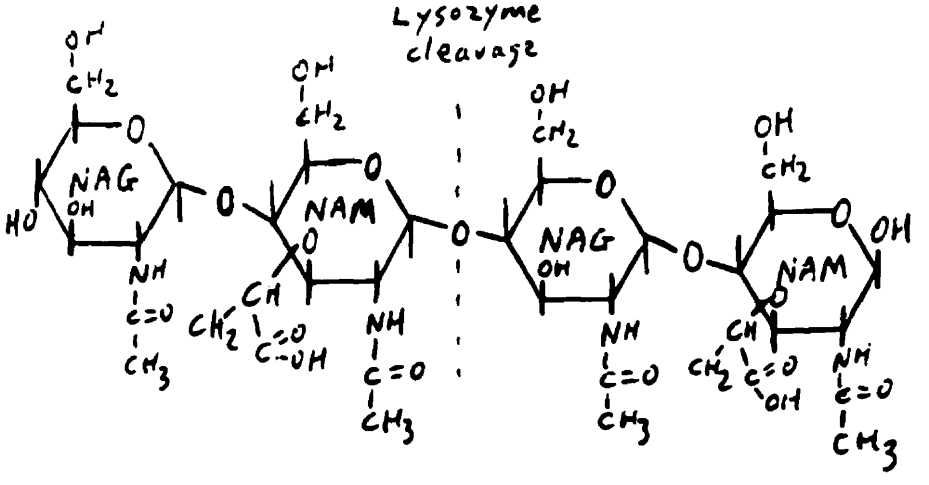

Figure 2.1. Site of lysozyme enzymatic cleavage of peptidoglycan. Peptidoglycan contains polymers of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) linked by β(1-4) glycosidic linkages. (Adapted from Proctor and Cunningham, 1988).

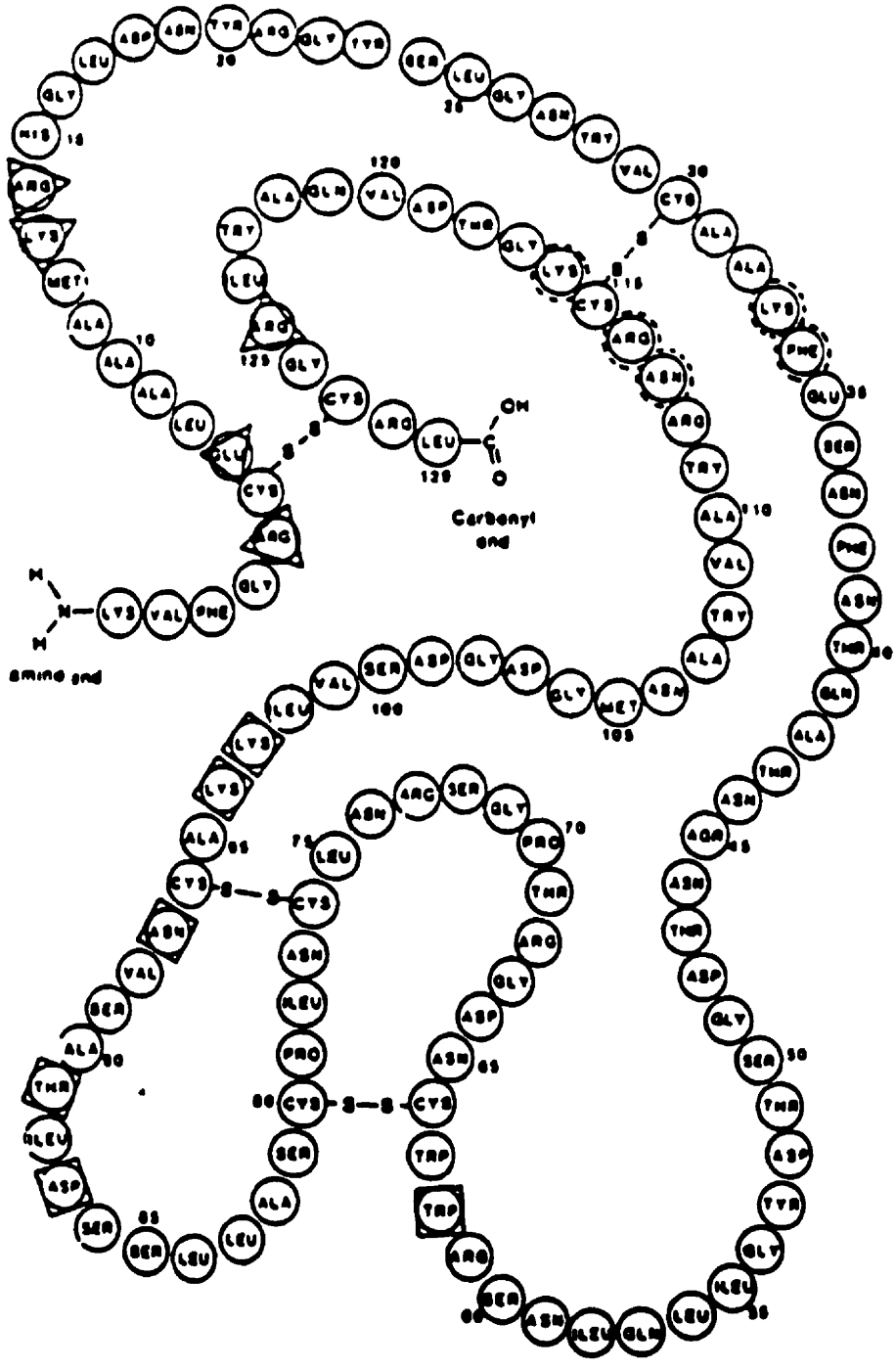

Figure 2.2. The two dimensional structure of lysozyme with the amino acids involved in the three antigenic sites indicated: site 1, triangle; site 2, square; and site 3, dotted circle. (Figure adapted from Proctor and Cunningham, 1988).

Research on lysozyme for food applications has centered on hen egg lysozyme (hereafter referred to as, lysozyme). Lysozyme is composed of a single 129 amino acid polypeptide chain containing four disulfide bonds, at least two of which must be present to maintain enzymatic activity (Fig. 2.2). The molecular weight of lysozyme is 14.3 - 14.6 kDa and its isoelectric point is 10.7 (Proctor and Cunningham, 1998).

The accepted method of assaying the enzymatic activity of lysozyme is by quantifying the loss in turbidity of a suspension of lyophilized *Micrococcus lysodeikticus* cells in phosphate buffer at 25°C. A decrease in absorbance at 450 nm of 0.001/min is taken as 1 unit (U) of enzyme activity (Proctor and Cunningham, 1988).

2.1.1. Conditions affecting lysozyme activity

The stability of lysozyme to thermal treatment is highly dependent upon the heating environment. Lysozyme is 50 times more stable when heated in phosphate buffer than in egg white and stability is pH dependent, with greater stability observed under acidic conditions than alkaline. The thermal stability of lysozyme may also be increased by the presence of NaCl or sugars (Proctor and Cunningham, 1998).

Davies et al. (1969) and Chang and Carr (1971) demonstrated that lysozyme is inactive in distilled water, and requires a minimum ionic strength for activity. Higher concentrations of cations are inhibitory and lysozyme activity is more sensitive to polyvalent than monovalent cations. The optimum ionic strength for lysozyme activity is pH dependent. At pH 7.0, 50 mM of a monovalent cation results in optimal activity while the same

concentration is inhibitory at pH 9.0 (Chang and Carr, 1971). Davies et al. (1969) theorized that the dependency of lysozyme activity on cation concentration was a consequence of the involvement of electrostatic forces in the interaction of lysozyme with the bacterial cell wall, and that the cations may be required for alignment of the active site of the enzyme with the substrate. However, high concentrations of polyvalent cations would interfere with interaction between positively charged lysozyme and a negatively charged cell wall. That cations have a role in forming an activated lysozyme/substrate complex was confirmed by Neville and Eyring (1972).

2.1.2. The use of lysozyme as an antimicrobial in food

Though many Gram positive organisms are sensitive to lysozyme, its application as an antimicrobial has been limited by resistance of Gram negative organisms whose cell wall is protected by the presence of the outer-membrane. A number of patents have been issued in Japan for the use of lysozyme as a component of preservative systems for application to a variety of vegetable, meat and fish products. These systems seem to be effective at controlling some lactic acid bacteria (LAB) (Proctor and Cunningham, 1988). Outside of Japan research has centered on the use of lysozyme to control butyric acid bacteria in cheese production (Proctor and Cunningham, 1988).

Hughey and Johnson (1987) investigated the sensitivity to lysozyme of 17 Gram positive and negative bacteria of concern to food spoilage or safety. The following organisms were observed to have some sensitivity to 200 mg/liter of lysozyme when growing in complex media; *Bacillus* (*B.*) *stearothermophilus*, *Clostridium* (*C.*) *botulinum* type B, *C.*

thermosaccharolyticum, *C. tyrobutyricum*, and *Campylobacter jejuni*. Inhibition of *B. cereus* was observed in static cultures but not shaken cultures. Bester and Lombard (1990), demonstrated that 250 U/ml of lysozyme (15000 U/mg) inhibited the growth of 2 isolates of *C. tyrobutyricum* and stimulated spore germination. The same study showed that 500 - 1000 U/ml reduced the growth rate of 3 of 4 *Lactobacillus* (*Lb.*) isolates and 3 of 4 coliform isolates from gouda cheese.

In the last decade a number of papers have been published on the use of lysozyme against *Listeria* (*L.*) *monocytogenes*. Smith et al. (1991) showed that reduction of growth temperature used for *L. monocytogenes*, increased the susceptibility of cells to lysis when suspended in buffer. Addition of 5000 U/ml of lysozyme (150,000 U/mg) to tryptose soy broth pH 5.5 at 5 °C increased the length of the lag phase from 10 to 20 days (Johansen et al., 1994). Kihm et al. (1994) found that *L. monocytogenes* was less sensitive to lysozyme in whole milk compared to growth media or phosphate buffer, though sensitivity was increased by heating at 55 °C. This study also showed that the protective effect of whole milk was associated with Mg^{2+} and Ca^{2+}.

2.1.3. Lysozyme interaction with other antimicrobial agents

Though lysozyme is ineffective against Gram negative bacteria, their resistance is not a consequence the unsuitability of the cell walls of these organisms as a substrate. Several researchers have examined the use of lysozyme with other antimicrobials, particularly membrane disrupting agents, in an effort to extend the range of bacteria that can be affected by lysozyme-based treatments.

Shively and Hartsell (1964a,b) demonstrated that the susceptibility of Gram negative pseudomonads to lysis by lysozyme can be increased by the use of the membrane disrupting agents, ethylene diamine tetraacetate (EDTA), sodium deoxycholate, isoamyl alcohol, ethanol, and thymol. Of the agents tested EDTA was the most effective when combined with lysozyme in a phosphate buffer system. Lysis was inhibited by acidic conditions or addition of NaCl, though the latter effect could be reversed by growth of cells on salt-containing media (Shively and Hartsell, 1964b). Expression of hen egg lysozyme in modified *E. coli* resulted in accumulation of lysozyme in the periplasmic space and cell lysis as a consequence of digestion of the cell wall (Fischer et al., 1993). The implication is that the lysozyme resistance of Gram negative organisms is a result of protection of the cell wall by the outer membrane.

Chelators have been a popular subject of investigation for use with lysozyme, as they may have an antimicrobial effect by limiting the availability of cations, and can act to destabilize the cell membranes of bacteria by complexing divalent cations which act as salt bridges between membrane macromolecules, such as lipopolysaccharides (Shelef and Seiter, 1993).

Razavi-Rohani and Griffiths (1996a,b) studied the effect of lysozyme combined with a variety of other agents using minimal inhibitory concentration (MIC) determination by the spiral gradient end point method against 7 Gram positive and 8 Gram negative organisms. Lysozyme in combination with EDTA was found to be more effective than either agent alone against the majority of the organisms tested and the MIC of the combination was reduced by lowering pH from 7 to 5 or by raising NaCl from 0.5 to 5% (Razavi-Rohani and Griffiths

1996a). Combination of lysozyme with sodium citrate, monoglycerol citrate, monolaurin, triglycerol laurate or butylated hydroxyanizole was not observed to result in increased inhibition compared to agents alone (Razavi-Rohani and Griffiths 1996a). The sensitivity of test organisms to monolaurin, triglycerol laurate or butylated hydroxyanizole alone, or with lysozyme was observed to be increased with lowered pH, (7 to 5), or increased NaCl, which suggests that antimicrobial effectiveness may be increased non-specifically by the presence of other growth restricting factors.

Since both lysozyme and the chelator, lactoferrin, are present in high concentrations in human polynuclear leucocytes and mucus, Ellison and Giehl (1991) speculated that they may act together against invading bacteria. They showed the combination could be effective against *Vibrio* (*V.*) *cholerae*, *Salmonella* (*S.*) *typhimurium* and *Escherichia* (E.) *coli*, and that lactoferrin activity is associated with interaction with the lipopolysaccharide . Payne et al. (1994) investigated the potential of lactoferrin or EDTA (≤ 2.5 mg/ml) combined with lysozyme (≤ 200 ug/ml) to control bacterial growth in ultra high temperature milk by a checker board assay. It was found that: the antimicrobials had no effect on *Pseudomonas* (*Ps*) *fluorescens* and *S. typhimurium*, the growth rate of *E. coli* was reduced by EDTA alone, and a combination of lysozyme and EDTA was more effective than either agent alone against *L. monocytogenes*. A combination of lactoferrin and lysozyme did affect the growth of *E. coli* and *L. monocytogenes*, but was less effective than EDTA.

El-Kest and Marth (1992a,b), showed that the susceptibility of *Listeria* strains to lysozyme could be increased by freezing and thawing and that this effect could be enhanced

by addition of lipase. Liberti et al. (1996) showed that a combination of lysozyme (0.3 mg/ml) and lipase (50 IU/ml) could restrict the growth of *L. monocytogenes* in broth at 5, 15 and 32 °C, depending on pH and NaCl concentration.

Park (1997) observed that the addition of a combination of lysozyme and papain or lysozyme and polysorbate 80 to vegetable juice pH 5.2 at 37 °C, had little effect on the growth of *E. coli*. However, if the vegetable juice was incubated for 2 h with 0.1% lysozyme and 1.0% papain, followed by addition of 0.01% polysorbate 80, *E. coli* numbers were reduced by 5 log CFU/ml.

It has been reported that use of trisodium phosphate as a chelator increased the sensitivity of a number of Gram negative organisms to lysozyme and nisin (Carneiro de Melo et al., 1998). However, the protocol used for the assessment appears flawed as the experiments were conducted in distilled water without stabilization of pH and no provision was made to protect cells from osmotic shock during serial dilution for plating.

2.1.4. Modification of lysozyme

Attempts have been made to extend the range of antimicrobial activity for lysozyme by chemical modification of the molecule. The modifications include conjugation with polysaccharides and fatty acids, altering the peptide chain of lysozyme and denaturation of the native lysozyme molecule. The aim of the majority of lysozyme modifications is to increase the hydrophobicity of the molecule to allow it to diffuse through the outer membrane to access the cell wall of Gram negative organisms.

Nakamura et al. (1990, 1991, 1992) produced a number of conjugates of lysozyme with attached polysaccharide groups. A conjugate of lysozyme and dextran with a molar ratio 1:1.8 and mass ratio of 1:9.4 was shown to have improved emulsifying and antimicrobial properties (Nakamura et al., 1990, 1991). Addition of 0.05% (protein w/v) of the lysozyme-dextran conjugate to cells suspended in phosphate buffer at 50 °C, increased the thermal death rate of the Gram negative bacteria *V. parahaemolyticus, E. coli, Aeromonas* (*A.*) *hydrophila, Proteus* (*P.*) *mirabilis* and *Klebsiella* (*K.*) *pneumoniae.* The lysozyme-dextran conjugate also increased the thermal death rate of Gram positive *B. cereus*, but the conjugate had no more effect than native lysozyme on *Staphylococcus* (*St.*) *aureus.* The residual enzyme activity of the conjugate was 13.3% of native lysozyme (Nakamura et al. 1990, 1991). A conjugate of lysozyme and galactomannan (molar ratio 1:1.7 and mass ratio 1:1.8) retained 80% of enzymatic activity (Nakamura et al., 1992). The galactomannan conjugate (0.05% protein w/v) produced faster thermal death rates at 50 °C in phosphate buffer of *V. parahaemolyticus, E. coli, P. mirabilis* and *K. pneumoniae.* The conjugate had no more effect than native lysozyme on *A. hydrophila.*

Lysozyme may also be modified by conjugation with fatty acids. (Ibrahim et al., 1991, 1993). Conjugates of lysozyme with palmitic acid were produced with 1 to 4 palmitic acid residues. The palmitic acid conjugates were assessed against *E. coli* cells suspended in phosphate buffer at room temperature for 10 min. Lysozyme with 4 palmitic acid residues (0.05% w/v) could reduce *E. coli* by 1 log CFU/ml, while the other conjugates with lower numbers of palmitic acid residues had lower activity. In the presence of 5 mM EDTA, lysozyme with 2 residues was the most effective and lysozyme with 3 or 4 residues was

progressively less effective. Ibrahim et al. (1993) also constructed conjugates of lysozyme with myristic or stearic acid to compare the effects of different fatty acid lengths on antimicrobial activity. Longer chain length was correlated with higher effectiveness against *E. coli* cells suspended in buffer, but of diacylated conjugates, palmitic acid was the most effective. The fatty acid conjugates were also shown to have improved ability to bind *E. coli* lipopolysaccharide, supporting the hypothesis that antibacterial activity against Gram negative organisms is due to increased interaction with the outer membrane.

Modified lysozyme molecules can be produced by expression of plasmids in transgenic organisms. Nakamura et al. (1993a,b) produced polymannosyl (71 kDa) and oligomannosyl (18 kDa) lysozyme by expresion of a plasmid in *Saccharomyces* (*S.*) *cerevisiae*. The oligomannosyl lysozyme possessed 73% of the enzymatic activity of native lysozyme, whereas the polymannosyl form possessed only 11% activity (Nakamura et al., 1993a). Both of the mannose lysozyme forms possessed superior emulsion properties compared to native lysozyme, but no information was reported on the antimicrobial activity of the modified lysozyme (Nakamura et al., 1993b). Ibrahim et al. (1992) produced modified lysozyme with 5 additional hydrophobic amino acids inserted into the C-terminus from *S. cerevisiae*. The enzymatic activity of the modified lysozyme was 80% of native lysozyme and possessed enhanced activity against *E. coli* cells suspended in phosphate buffer at 37 °C. A concentration of 2.5 μg/ml of the pentapeptide lysozyme reduced 5 log CFU/ml of *E. coli* cells to 49.5 % at pH 7 and 14.8% at pH 5 of the initial population. Native lysozyme at the same concentration reduced *E. coli* to 89.9% at pH 7 and 87.4% at pH 5 of the initial population.

Ibrahim et al. (1994) also modified lysozyme with the phenolic aldehyde, perillaldhehyde. Three derivatives with 0.5, 2.4 and 3.9 residues per lysozyme were produced. The enzymatic activity of the conjugates decreased with increasing residues, the 3.9 residue conjugate retaining 73% activity. Increasing the conjugate residues increased the bactericidal effect against both *E. coli* and *St. aureus* cells in phosphate buffer, but reductions greater than 1 log CFU/ml were not observed.

Outside of buffer systems, the only report of antimicrobial activity by palmitic acid or galactomannan conjugates of lysozyme is that by Nakamura et al., 1996. The authors reported that the feeding of either of these conjugates to live carp infected with *Edwardsiella tarda* increased the survival rate compared to control fish.

Valenta et al. (1997) produced a conjugate of lysozyme with 2.46 residues of hydrophobic cinnamaldehyde. The conjugate was intended for use as a preservative in cosmetics. Fifty µg/ml of the conjugate was capable of reducing both *E. coli* and *St. aureus* by 2 log CFU/ml in buffer at 37 °C.

In the last five years a number of papers have been published on hydrophobic heat-denatured lysozyme (Ibrahim et al., 1996a,b , 1997, 1998). Heating of lysozyme at 80 °C and pH 7 for increasing periods of time resulted in progressive irreversible denaturation of lysozyme by breaking of the interpeptide disulfide bonds, with concomitant loss of enzymatic activity (Ibrahim et al., 1996a). A concentration of 500 µg/ml of lysozyme heated for 20 min at 80 °C reduced viable *E. coli* cells suspended in buffer at 37 °C by 2 log CFU/ml, whereas

native lysozyme had no effect. The denatured lysozyme had no enzymatic activity but was equally effective as native lysozyme against *St. aureus* cells suspended in buffer (Ibrahim et al., 1996a). The heat-denatured lysozyme was shown to be 14 times more hydrophobic and to have a higher binding capacity for the membranes of *E. coli* and the peptidoglycan of *St. aureus* compared to native lysozyme (Ibrahim et al., 1996b). Further, the denatured lysozyme was demonstrated to be capable of permeabilizing liposomes made from *E. coli* phospholipids (Ibrahim et al., 1996b). Addition of divalent cations, Ca^{2+} and Mg^{2+} but not Na^{+}, inhibited the antimicrobial activity of the denatured lysozyme against *E. coli*. This effect was reversible by addition of EDTA, and Ibrahim et al. (1997) argued that this supported the hypothesis that the activity of the denatured lysozyme involved membrane interaction. Modification of the denaturing protocol to 20 min at 80 °C and pH 6.0 (80/6) preserved 50% of enzymatic activity (Ibrahim, 1998). The 80/6 lysozyme had an enhanced bactericidal effect against *St. aureus*, *E. coli* and *Salmonella enteritidis* suspended in buffer at 37 °C for 1 h. Treatment with 2500 ug/ml of 80/6 lysozyme reduced *St. aureus* cells by 4 log CFU/ml, compared to 3 log CFU/ml for native lysozyme . Native lysozyme at 2500 ug/ml had no effect on *E. coli* and *Salmonella enteritidis*, but 80/6 lysozyme at 500 ug/ml and 1000 ug/ml ,respectively, reduced these organisms by 5 log CFU/ml (Ibrahim et al., 1998). The 80/6 lysozyme was no more effective than native lysozyme against *B. subtilis* and *Ps. aeruginosa.*

2.2. Nisin

Nisin is one of a number of bacteriocins which are a diverse group of small antibacterial peptides produced by LAB and observed to possess activity against Gram positive bacteria, especially other LAB. Nisin consists of a single 34 amino acid peptide (3354

Da) produced by *Lactococcus lactis* ssp *lactis* (Fig. 2.3) (Delves-Broughton et al., 1996, Harris et al., 1992). Nisin belongs to the group of bacteriocins referred to as lantibiotics which contain the unusual amino acids, dehydroalanine, dehydrobutyrine, lanthionine and β-methyllanthionine. Nisin used for commercial purposes is nisin A, but another natural form is known which is called nisin Z in which histidine substitutes for asparagine at position 27 (Harris et al., 1992). It has been observed that nisin may form dimers or higher oligomers, but it is not known what significance these have in antimicrobial activity (Harris et al., 1992, Delves-Broughton et al., 1996).

The heat stability and solubility of nisin is dependent on pH (Delves-Broughton et al., 1996). Nisin can withstand prolonged heating at 121 °C at pH 2, but less stability is observed as pH increases in the range of 5 to 7. The solubility of nisin is maximal at pH 2.2 (56 mg/ml), and this is lowered to 3 mg/ml at pH 5 and 1 mg/ml at pH 11.

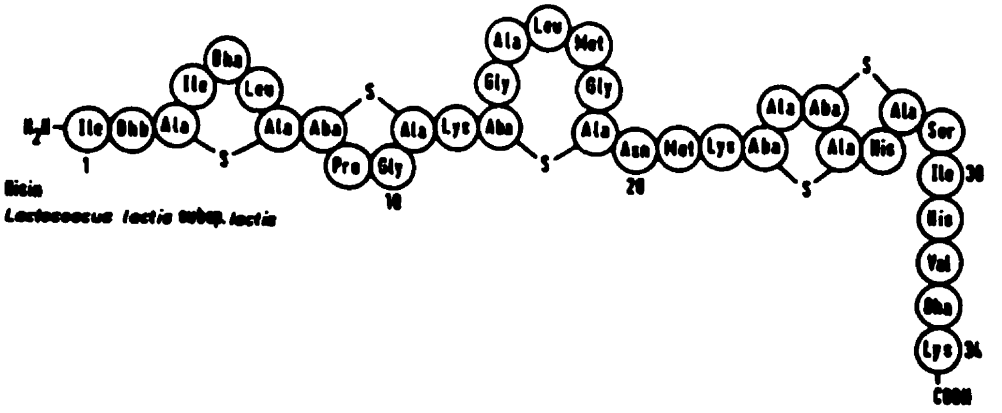

Figure 2.3. The two dimensional structure of nisin A. Dha, dehydroalanine; Dhb, dehydrobutyrine; Ala-S-Ala, lanthionine; Aba-S-Ala, β-methyllanthionine. (Figure adapted from Harris et al., 1992)

2.2.1. The mechanism of nisin activity

Nisin inhibits the outgrowth of spores and is bactericidal to the growing vegetative cells of sensitive species. Nisin activity against spores is based on a mechanism different from its action against vegetative cells. Morris et al. (1984) showed that nisin action against spores is dependent upon interaction with sulfhydryl groups of membrane proteins of newly germinated spores.

The mechanism of nisin activity against vegetative cells has been shown to involve alteration of the cell membrane resulting in leakage of low molecular weight cytoplasmic components and destruction of the proton motive force (PMF) (Bruno et al., 1992). This action is dependent upon interaction with cell membrane phospholipids Abee et al. (1994) observed that treatment of *L. monocytogenes* cells with nisin resulted in the loss of cellular K^+ and depolarization of the cytoplamic membrane. Hydrolysis and efflux of cellular adenosine triphosphate phosphate (ATP) was also observed.

Two models for nisin activity have been postulated. The "detergent-disruption" model is one in which the nisin molecule is postulated to disrupt the physical structure of the cell membrane in a manner similar to detergents. An alternative theory is the "poration complex" model which postulates the formation of an oligomeric pore in the cell membrane by the aggregation of nisin monomers to yield cytoplasmic leakage (Montville at al., 1995). The pore formation model is supported by experiments by van Kraaij et al. (1998) which demonstrated that the C-terminus of the nisin molecule translocates across model membrane vesicles. However, Breukink et al. (1998) observed nisin Z has an orientation parallel with the

membrane surface when inserted into a model phospholipid membrane, which is more consistent with a detergent disruptive action but may indicate that pore formation is transient and is dependent upon the proton motive force.

Nisin has been observed to stimulate oxygen consumption by glucose-metabolizing, non-growing *St. aureus* and *E. coli* cells to compensate for decreased proton motive force (Carneiro de Melo et al., 1996). This result indicates that nisin is capable of interacting with the cytoplasmic membrane of Gram negative organisms and that the outer-membrane does not protect cells from nisin by presenting a physical barrier to nisin.

2.2.2. Nisin resistance in sensitive bacteria

Nisin resistance in nisin producing strains is known to be plasmid mediated, though the mechanism is currently unknown. Some non-producer organisms have been identified as producing enzymes that break down nisin (Harris et al., 1992).

Nisin resistance may also be due to changes in cell structure in resistant strains. Analysis of the phospholipid content of the cell membrane of a nisin resistant strain of *L. monocytogenes* showed that though the types of phospholipids present were identical in a non-resistant strain, the nisin resistant strain possessed significantly lower amounts of phosphatidylglycerol, diphosphatidylglycerol and bis-phosphatidylglyceryl phosphate. Nisin resistant cells were observed to bind less nisin and have a less hydrophobic cell surface (Ming and Daeschel, 1995). Davies and Adams (1994) also observed reduced absorption of nisin to the cell membrane of resistant strains of *L. monocytogenes*, indicating a reduction in the

number or accessability of adsorption sites.

During an examination of two nisin resistant strains of *L. innocua*, evidence was found for changes in cell wall synthesis, though the target of nisin action is the cell membrane (Maisner-Patin and Richard, 1996). Resistant strains had cell walls that were observed by transmission electron microscopy to be thicker and rougher surfaced than the parent strain. Additionally, resistant strain cell walls were more hydrophobic and were observed to be more resistant to phages and the cell wall-targeting antibiotics, lysozyme, mutanolysin and vancomycin. It is possible that the observed changes to cell wall structure increased the ability of the cell wall to serve as a barrier to nisin.

Comparison of a nisin resistant mutant of *C. botulinum* with the wild type strain found no evidence for the production of a nisin degrading protein or changes to the protein content of the spore coat (Mazzotta and Montville, 1999). It was observed that the cell membrane of the resistant strain contained less (23%) unsaturated fatty acids, compared to the wild type (50%). The saturated fatty acid level in spores from both strains was similar (23%). However, the ratio of straight to branched chain saturated fatty acids in resistant spores was higher. Mazzotta and Montville (1999) suggested that nisin resistance was a consequence of the mutant strain possessing a less flexible cell membrane that resisted insertion of nisin.

De Martinis et al. (1997) investigated the effects of pH (5.0, 5.5 and 6.0), NaCl (0.5, 2.0 and 3.5% w/v) concentration and temperature (10, 20 and 30 °C) on the generation of nisin resistant strains of *L. monocytogenes* in nutrient broth. At 20 to 30 °C the rate of

production of resistant stains was independent of pH and salt content. At 10 °C, lowering pH and NaCl concentration reduced the frequency of nisin resistance.

2.2.3. The use of nisin as an antimicrobial in food

Nisin has been used as an antimicrobial in foods since the 1960's (for a review see Delves-Broughton et al., 1996). Nisin is most effective as an antimicrobial against LAB and other Gram positive organisms, notably spore formers such as bacilli and clostridia spp. and the pathogen *L. monocytogenes*. Research on nisin as an antimicrobial in foods has concentrated on applications against these sensitive organisms. Examples include addition of nisin to cheese and other dairy products to control clostridia, addition to low acid canned vegetables to prevent the growth of bacilli and clostridia, and addition to meat as a replacement for nitrite to control *C. botulinum*. Since yeasts are resistant to nisin, it may be used to control the growth of LAB contaminants in alcoholic fermentations. (Delves-Broughton et al., 1996).

Jenson et al. (1994) demonstrated that addition of 3.75 μg/g of pure nisin to crumpet batter could reduce numbers of *B. cereus* by >3.0 log CFU/g in packed crumpets. Zottola et al. (1994) manufactured Cheddar cheese using a nisin-producing starter culture and processed these into cheese spreads. Nisin-containing cheese spreads had a greater shelf life compared to controls when inoculated with *C. sporogenes*, *L. monocytogenes* and *St. aureus* cells. Inoculated bacteria were reduced over time when stored at 23 °C and 37 °C in both controls and nisin-containing cheese spreads, but numbers recovered dropped significantly faster in nisin-containing samples.

The nisin susceptibility of resistant organisms can be increased by the application of other restrictive conditions. Kalchayanand et al. (1992) demonstrated a greater bactericidal effect with 4000 IU(international units)/ml of nisin at 55 °C than heat or nisin alone against *Y. enterocolitica*, *Ps. fluorescens*, and *Ps. putida* suspended in 0.1% peptone. A combination of freeze/thaw and nisin had an increased bactericidal effect against *A. hydrophila*, *S. typhimurium*, *E. coli*, *Y. enterocolitica*, *Ps. fluorescens*, and *Ps. putida* (Kalchayanand et al., 1992). Addition of nisin (2500 IU/ml) to media broth inoculated with *S. enteritidis* reduced the time required for pasteurization at 55 °C by 35%, but the same treatment had a minimal effect in egg white or whole egg, though it was observed that nisin did increase the number of damaged cells as determined by recovery of samples on selective and non-selective media (Boziaris et al., 1998). A combination of heat treatment and nisin has also been demonstrated to have increased effectiveness against *L. monocytogenes* inoculated to canned lobster (Budu-Amoako et al., 1999). Thomas and Wimpenny (1996) observed that lowering temperature from 35 °C to 20 °C and increasing NaCl concentration (2.1 to 7 % w/v) increased the effectiveness of nisin against *St. aureus* growing on gradient plates. Temperature within the range of 20 °C to 35 °C was observed to have no effect on nisin activity against *L. monocytogenes* but increasing NaCl increased nisin inhibition. Nisin inhibition of both organisms was increased by decreasing pH from 7.9 to 5 (Thomas and Wimpenny, 1996).

Milk fat may protect *L. monocytogenes* from nisin. Jung et al. (1992) observed that increased fat content reduced the effectiveness of nisin against *L. monocytogenes* Scott A and Jalisco in milk at 37 °C. It was also demonstrated that nisin activity could be increased by the addition of the non-ionic detergent, tween-80, and this could counteract the protective effect

of fat. However, anionic lecithin had no effect on nisin activity (Jung et al., 1992). Addition of 14 mg/kg of nisin to ice cream mix, with 3% fat, resulted in the reduction of inoculated *L. monocytogenes* from approximately 3.25 log CFU/ml to less than 0.5 log CFU/ml after 14 days storage at -18 °C, the same reduction took 84 days storage with 10% fat (Dean and Zottola, 1996).

Nisin has been adsorbed to hydrophilic and hydrophobic silicon surfaces in an attempt to develop a surface treatment system for targeting microbial biofilms in food production areas (Daeschel et al., 1992). The numbers of attached *L. monocytogenes* cells remained static on nisin-treated surfaces, whereas bacteria freely increased on nisin free or heat-inactivated, nisin-treated surfaces. Staining with iodonitrotetrazolium revealed that 95% of attached cells were viable in controls as opposed to 25% viability on nisin- treated surfaces (Bower et al., 1995).

Mahadeo and Tatini (1994) used a solution of 100 U/ml of nisin against *L. monocytogenes* cells in buffer suspension or against cells attached to squares of turkey skin. Cells in suspension were reduced by >4 log CFU/ml, while the same treatment applied to cells attached to squares of turkey skin produced only a 1 log CFU/ml reduction.

Nisin has been observed to reduce populations of *L. monocytogenes* on cooked pork. Fang and Lin (1994) studied the effect of modified atmosphere packaging (MAP) of cooked pork dipped in nisin (10^4 IU/ml) on the growth of *L. monocytogenes* and *Ps. fragi*, and found that while nisin had a bactericidal effect on *L. monocytogenes*, and that while MAP restricted

the growth of *Ps. fragi* there was no evidence for an interaction between the treatments against either organism.

The effectiveness of nisin treatment of meat has been shown to be increased by immobilizing the nisin in a gel. Cutter and Siragusa (1997) showed that 0.1 mg/ml pure nisin incorporated into a calcium alginate gel was more effective against *B. thermosphacta* growing on ground beef than unbound nisin. Fang and Lin (1995) demonstrated greater reduction of *L. monocytogenes* with a nisin treatment of cooked pork tenderloins when the nisin was immobilized in a calcium alginate gel. These studies also demonstrated that the activity of nisin in the meat persisted longer when immobilized in alginate gel. Nisin added to ground pork at 300, 1500 and 3000 IU/ml has been shown to have a bactericidal effect on *L. innocua*; however, in all cases the population of surviving cells started to increase at a rate similar to controls after 2 days storage at 4 °C, coincident with the end of lag phase in controls (Murray and Richard, 1997).

Rose et al. (1999) suggested that nisin inactivation may occur in fresh meat by interaction of nisin with glutathione, catalyzed by glutathione-S-transferase; a reaction that could occur when mixed in product prior to cooking. However, this reaction cannot be the sole cause of nisin inactivation in meat, as the activity of nisin was reduced over time at 4 °C in cooked pork (Fang and Lin 1995).

2.2.4. Nisin interaction with other antimicrobial agents

Experiments carried out on bacterial cells in buffers has shown that a combination of

nisin and a chelator may be more effective at killing Gram negative bacteria than either agent alone.

Stevens et al. (1991) showed a variety of Gram negative bacteria including 20 *Salmonella* spp., *Enterobacter aerogenes*, *Shigella flexneri*, *Citrobacter freundii*, and *E. coli* O157:H7 experienced reductions of 3 to 7 log CFU/ml when subjected to 50 μg/ml nisin and 20 mM EDTA when suspended in buffer at 37 °C for 30 min to 1 h. A later paper extended this work by studying the effects of different chelators, temperature and nisin concentration (Stevens et al., 1992). EDTA and citric acid were the most effective chelators, compared to ethylene bis tetraacetic acid (EGTA) and dibasic sodium phosphate. Reduction of cells was increased with increasing nisin and temperature from 4 to 42 °C. Similar results were observed by Cutter and Siragusa (1995b), who also demonstrated that the addition of Mg^{2+} and Ca^{2+} had an inhibitory effect on the antimicrobial activity. Schved et al. (1994) observed a 4 log CFU/ml reduction of *E. coli* and a 3 log reduction of *S. typhimurium* cells suspended in Tris buffer at 30 °C with 3200 U/ml nisin and 20 mM EDTA; no reduction was observed with either agent alone. Addition of Mg^{2+} and Ca^{2+} was again observed to prevent cell death, supporting the theory that chelator disruption of the outer membrane allowed nisin access to the cytoplasmic membrane.

Applications of nisin/chelator-based antimicrobial treatments towards organisms attached to the surface of meat have resulted in less spectacular results against Gram negative organisms than observed in buffer systems. Treatment of beef cubes with 50 μg/mL of pure nisin and 50 mM EDTA reduced attached *S. typhimurium* or *E. coli* O157:H7 by less than

0.5 Log CFU/cm^2 (Cutter and Siragusa, 1995a). When beef was treated with solutions of 2% (w/v) lactic acid or 2% (w/v) polylactic acid alone, or in combination with 200 IU/ml of pure nisin, a bactericidal effect was observed against the *Enterobacteriaceae* and *Pseudomonas* spp. Addition of nisin did not increase the effectiveness of the acid treatments, and none of the treatments affected the numbers of LAB recovered (Ariyapitipun et al., 1999). Sheffet et al. (1995) demonstrated that immersion of turkey skin for 30 min in a solution of 100 μg/ml nisin, 5 mM EDTA, 3 % (w/v) citric acid and 0.5 % Tween 20, resulted in a 4.9 log CFU/ml greater reduction of attached *S. typhimurium* cells compared to 20 ppm chlorine. Application of the same treatment to whole turkey drumsticks increased the shelf life by 1.5 to 3 days at 4 °C (Sheffet et al., 1995). Treatment of vacuum packed beef at 4 °C with 5000 IU/ml nisin or 20 mM EDTA, alone or combined had no effect on *E. coli* O157:H7 inoculated at log 3 CFU/cm^2 (Zhang and Mustapha, 1999). *L. monocytogenes* on beef subjected to the same treatment were reduced 2 log CFU/cm^2 by nisin and 1 log CFU/cm^2 by nisin and EDTA. Since the numbers of *L. monocytogenes* recovered from controls and treated beef was constant over the storage period of 30 days, no conclusions can be drawn about the effects on a population of cells capable of recovery (Zhang and Mustapha, 1999).

Padgett et al. (1998) investigated the possibility incorporating nisin with EDTA, or lysozyme with EDTA into edible films manufactured from soy protein or corn zein. It was observed that the inhibition zones of *Lb. plantarum* generated by the films were smaller when the films were heat-pressed as opposed to cold cast, indicating that heating reduced the antimicrobial activity.

As an alternative to chelators, Zapico et al. (1998) examined the use of nisin with lactoperoxidase and demonstrated that the combination interacted synergistically to reduce *L. monocytogenes* in UHT milk, but the system was not investigated for increased activity against Gram negative organisms.

2.3. Combinations of lysozyme and nisin

The only study published to date on the use of both lysozyme and nisin in an antimicrobial treatment was a study by Hauben et al. (1996) on the effect of these agents and EDTA against *E. coli* subjected to high pressure treatments. The authors observed that application of increasing pressure in the range of 220 to 320 MPa resulted in increased lethality of *E. coli* cells suspended in phosphate buffer. The addition of lysozyme, nisin and EDTA, alone, paired or all three simultaneously, resulted in greater reductions of *E. coli* under pressure. At 320 MPa of pressure, the treatment was sufficient to cause a 4.06 log CFU/ml reduction of *E. coli*, while addition of the antimicrobial agents produced log reductions of, 5.46 (lysozyme), 5.71 (nisin), 6.05 (EDTA), 6.90 (lysozyme + nisin), 7.92 (lysozyme + EDTA), 8.22 (nisin + EDTA) and >8.22 (lysozyme + nisin +EDTA). Since high pressures disrupt the membrane of bacterial cells these results can be considered to provide some support for the hypothesis that membrane disruption can enhance lysozyme and nisin activity against Gram negative bacteria. However, high pressures have complex effects on the macromolecules that comprise bacterial cells beyond membrane disruption.

2.4. Summary

A review of the current literature on lysozyme and nisin indicates that a number of issues still have be resolved in the development of new preservative systems based on these peptides. The mechanism of nisin activity still has to be fully elucidated. Though nisin may be used as a preservative without complete understanding of its mechanism of action, more complete knowledge would perhaps permit rational development of methods to extend its activity range.

The same argument can be made regarding lysozyme. The work of Ibrahim and co-workers (Ibrahim et al, 1996a, 1996b, 1997; Ibrahim 1998) on heat-denatured lysozyme indicate that its antimicrobial activity may not be solely due to its enzymatic activity. Further, identification of any cofactors involved in lysozyme activity in animal tissues and secretions may provide a basis for development of systems for use in food.

A significant body of work has been produced demonstrating that combinations of lysozyme or nisin with other antimicrobials have increased activity against Gram negative bacteria. Conclusions on the suitability of these systems for use in food products should be drawn with caution, as the great majority of these studies were conducted against cells suspended in buffer systems rather than on growing cells in nutrient rich environments, as are found in foods. Similar criticism may also be made of the methods used to assay the antimicrobial activity of modified lysozyme, though the modification of lysozyme appears to be a promising line of research.

It should be noted though that a number of studies have demonstrated that addition of lysozyme or nisin to specific food systems may be successful in controlling specifically targeted sensitive organisms, such as *Listeria* or *Clostridium* species.

3. Evaluation of antimicrobial combination treatments using response surface analysis of checker board experiments in nutrient broth.

3.1. Abstract

Experiments were conducted in nutrient broth to determine an effective antimicrobial treatment for use in cured meat products utilizing lysozyme in combination with nisin, chelators (EDTA and tripolyphosphate) and diacetyl. The tests were conducted against a variety of Gram positive and negative organisms that are of concern for safety or spoilage reasons in cured meat products. The results were statistically analyzed to determine if interactions occurred between lysozyme and other agents and to determine if lysozyme potentiated the action of any of the other antimicrobials.

Potentiation of lysozyme activity was observed in two cases: lysozyme with nisin against *Brochothrix thermosphacta* and lysozyme with EDTA and diacetyl against *Staphylococcus aureus*. The trials did not indicate potentiation of lysozyme activity in any of the other combinations tested.

3.2. Introduction

A body of work has been produced regarding the use of chelators or other membrane disrupting agents such as polysorbate 80 and lactoferrin, with lysozyme (Hughey and Johnson, 1987; Ellison and Giehl, 1991; Payne et al., 1994; Park, 1997) or nisin (Stevens et al., 1991, 1992; Cutter and Siragusa, 1995ab) to achieve enhanced antimicrobial activity against food borne bacterial pathogens or food spoilage bacteria. The use of a membrane

disrupting antimicrobial in combination with lysozyme or nisin has been hypothesized to be more effective than the application of these agents alone since they target different cell structures (Stevens et al., 1991).

Lysozyme is a 14.6 kDa single peptide (Proctor and Cunningham, 1988), which possesses enzymatic activity against the β(1-4) glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine found in peptidoglycan. Gram positive bacteria are more sensitive to lysozyme activity than Gram negative bacteria which are highly resistant (Proctor and Cunningham, 1988).

Nisin is a bacteriocin produced by the lactic acid bacteria (LAB), *Lactococcus lactis* (Delves-Broughton et al., 1996). Nisin activity involves alteration of the cell membrane of sensitive organisms resulting in the leakage of low molecular weight cytoplasmic components and destruction of the proton motive force (PMF) (Bruno et al., 1992).

The antimicrobial effect of chelators, such as tripolyphosphate and ethylenediamine tetraacetate (EDTA), is due to destabilization of the cell membrane of bacteria by complexing divalent cations which act as salt bridges between membrane macromolecules, such as lipopolysaccharrides (for reviews see, Varaa, 1992; Shelef and Seiter, 1993).

Diacetyl was also included in these experiments as it has been demonstrated to be an effective antimicrobial against a wide range of Gram negative and Gram positive bacteria, although LAB are generally resistant (Jay, 1982a, 1982b; Jay et al., 1983; Jay and Rivers,

1984). The resistance of LAB to diacetyl was seen as potentially adventitious. In vacuum packaged cured meat products LAB extend self life and there was some concern that the use of lysozyme and nisin could inhibit LAB but be ineffective against Gram negative bacteria. The antimicrobial mechanism of diacetyl is unknown though Jay et al. (1983) suggested that it may result from inhibition of arginine metabolism.

In this study we conducted experiments, in nutrient broth to evaluate the potential effectiveness of lysozyme, used alone and in combination with other agents, as an antimicrobial for use in cured meat products to enhance shelf life and product safety.

The combinations of agents evaluated in this study with lysozyme included: nisin, nisin and EDTA, nisin and tripolyphosphate, nisin and diacetyl, EDTA and diacetyl. The organisms tested were, *Brochothrix thermosphacta*, *Escherichia coli* O157:H7, *Lactobacillus sakei*, *Lactobacillus curvatus*, *Leuconostoc mesenteroides*, *Listeria monocytogenes*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Salmonella typhimurium*, *Serratia grimesii*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Weissella viridescens* (*Lactobacillus viridescens*). The organisms chosen were selected as representative organisms of spoilage or safety concern, which may be found composing the flora of cured meat products.

3.3. Materials and Methods

3.3.1. Cultures and Incubation Conditions

B. thermosphacta B2 was from Dr G. Greer Agriculture and Agrifood Canada (AAFC) Research Station, Lacombe, AB; BHI; aerobic. *E. coli* O157:H7 strain E318 was a

human clinical isolate provided by Dr. R. Johnson, Health Canada, Guelph , ON; BHI; aerobic. *Lb. sake* #7, *Lb. curvatus* #15 and *Lc. mesenteroides* #11, were isolated from spoiled cured meats (Holley et al., 1996); APT; anaerobic. *Listeria monocytogenes*, University of Manitoba, Department of Food Science culture collection; BHI; aerobic. *P. acidilactici* 122P and *P. pentosaceus* 116P, were isolated from commercial meat starter cultures; APT; anaerobic. *S. typhimurium* #98 was from the AAFC collection, Guelph ON; BHI; anaerobic. *Ser. grimesii* S12 was isolated from cooked roast beef, AAFC collection, Summerland, BC; BHI; anaerobic. *Sh. putrefaciens*, was from the ATCC as #8071; BHI, aerobic. *St. aureus* # 427 was from the AAFC collection, Guelph ON; BHI; aerobic. *En. faecalis* #28 (ATCC #7080), was from the AAFC collection, Guelph, ON; BHI; anaerobic. *W. viridescens* # 13 (ATCC # 12706), was from the AAFC collection, Guelph ON; APT; anaerobic.

Cultures were transferred to fresh agar slants at 4 °C monthly and stored as frozen glycerol stocks. Cultures were streak-plated once a week and cultures for experiments were inoculated into media from a single colony and incubated overnight under the appropriate atmospheric conditions. All cultures were maintained in either APT or BHI media, except for *Lb. sake* #7 which was maintained on M17 agar but grown in APT. Incubations were conducted at room temperature, (24 °C).

3.3.2. Materials

Lysozyme HCl (lot# A7333-F) and nisin (25 g/Kg w/w Chrisin, lot# 17127) were provided by Canadian Inovatech, Abbotsford, BC. Morpholinoethane sulfonic acid, MES (2-4-morpholino-ethane sulfonic acid), EDTA (disodium ethylenediamine tetraacetate) and

tripolyphosphate (sodium phosphate tripoly) were obtained from Fisher Scientific, Toronto, ON. Diacetyl (2,3-butadione) was from Aldrich Chemicals, Oakville, ON. The APT broth (all purpose tween broth) was from BBL, Becton-Dickinson, Franklin Lakes, NJ . The BHI broth (brain heart infusion broth) and granulated agar, were from DIFCO, Detroit, Michigan. The M17 broth was from Oxoid, Nepean, ON. GasPak Jars (BBL, Becton-Dickinson) were made anaerobic using the GasPak Plus Anaerobic System with palladium catalyst. An anaerobic incubator (National Appliance Co., Portland, Oregon) flushed 3 times with CO_2 was also used for anaerobic incubations. Growth was monitored in 96 well polypropylene micro-titre plates (Corning-Costar, Corning Incorporated, Acton, MA), using a Titretek Multiskan MCC/340 Mk II type 347 spectrophotometer (Flow Laboratories International SA, Switzerland), at a wavelength of 450 nm.

3.3.3. Methods

The protocol described here was adapted from that of Maclean et al. (1997a,b). The media used in these experiments were, ATP broth or BHI broth, both were buffered with 10g.L^{-1} morpholinoethane sulphonic acid (MES) (Buncic et al., 1995) and adjusted to pH 6.0 with 0.1 mol/L HCl and NaOH.

Each well of a sterile 96 micotitre plate, one for each organism and combination to be tested, was filled with 100 µL of APT broth or BHI . Lysozyme, nisin and lysozyme-nisin mixes were prepared freshly (1 mg/mL), dissolved in previously sterilized broth. EDTA, tripolyphosphate and diacetyl solutions were also prepared fresh each day. Stock solutions of EDTA or tripolyphosphate were prepared in distilled water and added to previously

autoclaved broth media following filter sterilization to achieve the desired final concentration. Diacetyl was weighed directly into sterilized broth to achieve a concentration of approximately 1000-1200 mg/L. The concentration of the agents prepared was recorded. When necessary, agents were prepared together in the same beaker to facilitate testing of three way interactions.

The first column of wells in each microtitre plate received 100 µL of broth media containing the first agent to be tested. After mixing, 100 µL was transferred to the second column of wells. The process of serial dilution was repeated until the last column, #12, which was left without the first agent. The first row, #A, of wells received 100 µL of solution containing the second agent to be tested, which was mixed and 100 µL transferred to the second row of wells. The process was repeated until the last row, #H, which was left without the second agent.

Each bacterium to be tested was inoculated, from a single colony, into 5 mL of APT or BHI broth in a test tube and incubated overnight. Incubation was at room temperature under aerobic or anaerobic conditions, as appropriate. Serial 1/100 dilutions (50 µl in 5 mL) of overnight culture were prepared in broth to 10^{-6}. The inoculum was enumerated by plating the 10^{-6} dilution on to media plates which were incubated for two days and then counted. Each well in the micro-titre plates was inoculated with 10 µL of 10^{-4} dilution of the culture to be tested. The well #H12 served as a positive control and the row #H and column #12 indicated the minimum inhibitory concentration (MIC) of each agent alone. The inoculated micro-titre plates were then incubated for 60 hours at 24 °C, under the appropriate

atmospheric conditions. At the end of incubation, the wells were scored visually as growth or no growth. The concentration of the agents in the last wells in the row or column in which growth did not occur were recorded for data analysis.

3.3.4. Statistical Analysis

For each combination of agents that an organism was subjected to, the minimum concentration at which inhibition of growth occurred was recorded. These inhibitory concentrations were used to generate a response surface model (Hill and Hunter, 1966; Box et al., 1978) by treating the inhibitory concentrations as points along a curve (2 agents) or surface (3 agents) that describes the interface between inhibitory and non-inhibitory concentrations of agents. The response surface model was developed using the JMP IN statistics program (Sall and Lehman, 1996). The response surface model was an effect-screening model in which lysozyme concentration is determined to be the response and the surface is described by the function:

$$y = a + bx + cz + dx^2 + ez^2 + fxz$$

Where: y = lysozyme concentration (mg/L)
a= y intercept (mg/L)
x = agent x concentration (mg/L)
z = agent z concentration (mg/L)
b, c, d (L/mg), e (L/mg), f (L/mg), = model parameters for each effect
A representative model and contour plot is presented in Figure 3.1.

The program calculated a value Prob>F, an F test value which can be used to evaluate the probability of the significance of individual agents (effects) contribution to the response. Prob>F is the "probability of being wrong if you declare an effect to be non null" (Sall and

Lehman, 1996), This can be interpreted as the probability that the data entered does not indicate an interaction between lysozyme and the other agent. The apparent presence of an interaction between lysozyme and another agent was determined using an alpha value of 0.10. In other words, it was determined that no interaction was present when there was a greater than 0.10 probability of being wrong if the null hypothesis (no interaction) were rejected. This form of analysis does not indicate the type of interaction occurring, it merely indicates the probability of interaction. The characterization of the interaction as additive, antagonistic or synergistic remains with investigator. An example of a contour plot generated from a model is shown in Figure 3.1.

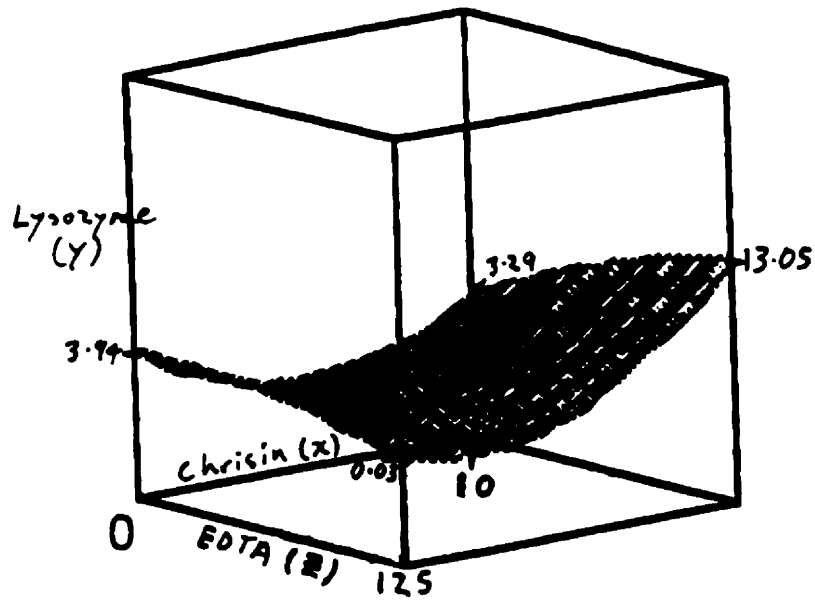

Figure 3.1. Contour plot of a response surface model generated for the activity of lysozyme, nisin and EDTA against *B. thermosphacta* at 24 °C. The model function is:

$$y = a + bx + cz + dx^2 + ez^2 + fxz$$

The model parameters are: y = lysozyme mg/L, x = nisin mg/L, z = EDTA mg/L, a = 3.950 mg/L, b = -2.237, c = 0.00344, d = 0.2171 L/mg, e = -0.0003 L/mg, f = 0.0110 L/mg.

The entry "lost d.f." was made in Table 3 and 5 for two of the combinations tested: *E. coli* 0157:H7 with lysozyme/nisin/EDTA, and *L. curvatus* with lysozyme/diacetyl/EDTA, respectively. The data for these combinations did not possess enough variability to allow calculation of a Prob>F. It can be concluded that no interaction occurred in these instances

3.4. Results

The MICs required for inhibition of growth for the various agents alone are reported in Table 3.1 as parts per million. Where no inhibition of growth was observed the highest concentration of agent tested was reported. Of the organisms tested, only *B. thermosphacta* B2 was inhibited by concentrations of lysozyme of 500 mg/L or less. Nisin was effective against all of the Gram positive organisms tested at varying concentrations. Nisin was ineffective against all of the Gram negative organisms at the concentrations tested. Of the two chelators tested, no inhibition was observed with tripolyphosphate at the concentrations tested, however, EDTA was inhibitory against most of the organisms tested. The only agent tested that was effective against both *E. coli* and *Salmonella* was diacetyl at 250 mg/L. *Serratia grimesii* was resistant to all of the treatments in this series of experiments. The MIC of lysozyme and lysozyme:nisin::1:3 mix was also determined in the presence of sodium nitrite to determine if the presence of this chemical enhanced or inhibited the activity of lysozyme. No effect upon the MIC of lysozyme or lysozyme:nisin::1:3 was observed with nitrite levels of 40, 80, and 160 mg/L.

In Tables 3.2 to 3.14 the results of statistical analysis for the combination treatments are reported, for each organism independently. The results are reported as: n, the number of

data points on which the model was based, not the number of experimental replicates, (where no inhibition of growth was observed there where no data points and the entry "---" is made); effect, the agents whose interaction is being tested; Prob>F, the probability of the null hypothesis being true, no interaction. Where interaction was observed it was characterized as positive or negative. A determination of positive interaction was made if increasing the concentration of the agent consistently reduced the concentration of lysozyme required to achieve inhibition. A negative interaction was determined where increasing the concentration of the agent consistently increased the concentration of lysozyme required to achieve inhibition. Where interaction was not observed it can be concluded that inhibition occurred as a result of the presence of a limiting concentration of only one of the agents present.

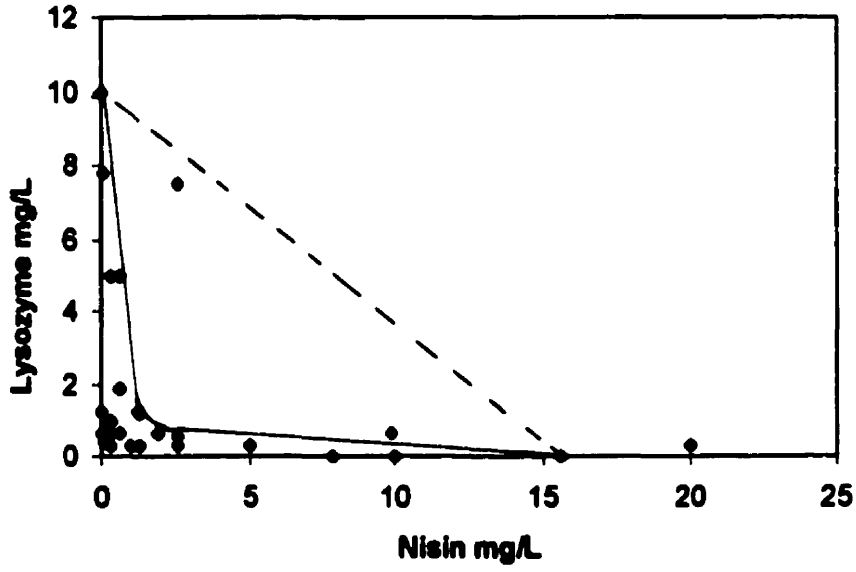

Figure 3.2. Inhibitory concentrations of lysozyme and nisin combination treatments against *Brochothrix thermosphacta*. The dotted line drawn between minimum inhibitory concentration values for the two agents alone, indicates the expected result of an additive interaction. A concave curve below this line indicates a synergistic interaction. A convex curve above the line indicates an antagonistic interaction.

The results of the statistical analysis indicated an interaction only between lysozyme and nisin with *B. thermosphacta* B2 (Table 3.2). The interaction between these two agents was apparently positive (Fig. 3.2). The only other apparent interaction observed was a three way interaction between lysozyme, EDTA and diacetyl in the treatment of *S. aureus* (Table 12). The lowest effective inhibitory concentrations of all three agents in combination was 120 mg/L lysozyme / 31 mg/L diacetyl / 62.5 mg/L EDTA. *S. aureus* was resistant to lysozyme and diacetyl alone or in combination. The MIC of EDTA against *S. aureus* was 125 mg/L.

3. 5. Discussion

Previous studies have reported observation of antimicrobial action by lysozyme (Shively and Hartsell, 1964a, 1964b; Hughey and Johnson, 1987; Ellison and Giehl, 1991; Payne et al., 1994; Park, 1997) or nisin (Stevens et al., 1991, 1992; Cutter and Siragusa, 1995a,) against Gram negative and Gram positive organisms in the presence of membrane disrupting agents.

The majority of these reports are based upon observations made with organisms suspended in a buffer rather than growing in nutrient media (Shively and Hartsell, 1964a, 1964b; Ellison and Giehl, 1991; Stevens et al., 1991, 1992; Cutter and Siragusa, 1995b). Where studies have been conducted in media capable of supporting growth, no activity against Gram negative organisms has been reported except where unrealistically high levels of agents for food applications have been used. Park (1997) claimed lysozyme activity against *E. coli* in nutrient broth and vegetable juice, at levels of 1 to 4% w/v. Cutter and Siragusa (1995b) reported antimicrobial activity of nisin with chelators against *E. coli* and *Salmonella*

in buffer, but reported no significant effect upon the same organisms when trials were conducted on beef (1995a).

The effectiveness of lysozyme and nisin in potentiating antimicrobial effects when used in conjunction with chelators appears to be only effective under restrictive growth conditions. The absence of potentiation effects under less restrictive conditions may indicate the ability of the organisms to recover from damage inflicted by the antimicrobials or protection effects occurring in complex media. Since food products are nutrient rich complex environments, evaluation of antimicrobials under nutrient deficient conditions would appear to be of limited value.

The results of these experiments do not indicate a potentiation of lysozyme in combination with the other agents tested, against the tested organisms. At this point it may well be valuable to reassess the strategy to be followed for the application of lysozyme combination treatments in cured meat products. The bacteria-specific nature of the enzymatic activity of lysozyme makes it of continuing interest in seeking new antimicrobial treatments.

It has been suggested that the protection from lysozyme treatment observed in complex media is a related to charge effects from the presence of ions which interfere with the ability of lysozyme to form close associations with the cell wall (Davies et al., 1969). Additionally, the presence of numerous freely available ions in complex media can also be expected to reduce the membrane disruption effect of chelators. Successful application of lysozyme to the treatment of cured meat products may require the use of some other method

to ensure lysozyme is able to form close association with the cell wall of target organisms.

Previous research has demonstrated the antimicrobial action of chemically modified lysozyme against Gram negative organisms (Nakamura et al., 1996, 1992, 1991; Ibrahim et al., 1992, 1991). In these studies the action of lysozyme was tested against such organisms as *E. coli*, *Vibrio parahaemolyticus*, *Proteus mirabilis*, *Klebsiella pneumoniae* and *Edwardsiella tarda* after conjugation with galactomannan or palmitic acid. The aim of these conjugations was to increase the lipid solubility of the molecule, to allow lysozyme to penetrate the outer membrane and act enzymatically upon the cell wall. Though this line of research has not been investigated in the context of application to cured meat products it could provide an alternative approach to developing a successful antimicrobial treatment based upon the enzymatic activity of lysozyme.

Table 3.1. Minimum inhibitory concentration of single agents tested in mg/L against organisms incubated in broth cultures at 24 °C.

Organism	Lysozyme	Nisin	EDTA	Diacetyl	Tripoly-phosphate
Brochothrix thermosphacta	10	5.6	250	>500[1]	>4000
Enterococcus faecalis	>500	500	500	>500	>4000
Escherichia coli	>500	>500	1000	250	>4000
Lactobacillus curvatus	>500	5	1000	>500	>4000
Lactobacillus sakei	>500	500	500	>500	>4000
Leuconostoc mesenteroides	>500	1.3	2000	>500	>4000
Listeria monocytogenes	>500	250	250	>500	>4000
Pediococcus acidilactici	>500	0.63	500	>500	>4000
Pediococcus pentosaceus	>500	5	1000	>500	>4000
Salmonella typhimurium	>500	>500	>2000	250	>4000
Serratia grimesii	>500	>500	>2000	>500	>4000
Shewanella putrefaciens	>500	>500	>2000	NT[2]	>4000
Staphylococcus aureus	>500	250	250	>500	1000
Weissella viridescens	>500	7.8	1000	>500	>4000

1. Where ">" is used, the number following represents the highest concentration tested.
2. NT - not tested

Table 3.2. Results of combination treatments against *Brochothrix thermosphacta* grown at 24 °C in BHI broth, aerobically.

Combination	n	Effect	Prob>F	Interaction $\alpha = 0.10$	Type of Interaction
lysozyme/nisin	40	nisin	0.0589	yes	positive
lysozyme/nisin /EDTA	65	nisin	0.0262	yes	positive
		EDTA	0.9481	no	
		nisin*EDTA	0.7225	no	
lysozyme/nisin /diacetyl	55	nisin	0.0276	yes	positive
		diacetyl	0.3431	no	
		nisin*diacetyl	0.6398	no	
lysozyme/nisin /tripoly PO_4	51	nisin	0.0316	yes	positive
		tripoly PO_4	0.3381	no	
		nisin*tripoly PO_4	0.3005	no	
lysozyme/EDTA /diacetyl	24	diacetyl	0.1206	no	
		EDTA	0.2327	no	
		diacetyl*EDTA	0.5820	no	

Table 3.3. Results of combination treatments against *Escherichia coli* O157:H7 grown at 24 °C in BHI broth, aerobically.

Combination	n	Effect	Prob>F	Interaction $\alpha = 0.10$
lysozyme/nisin	---	nisin	No activity	
lysozyme/nisin /EDTA	37	nisin EDTA nisin*EDTA	lost d.f.	no
lysozyme/nisin /diacetyl	58	nisin diacetyl nisin*diacetyl	0.3901 0.9811 0.9847	no no no
lysozyme/nisin /tripoly PO_4	---	nisin tripoly PO_4 nisin*tripoly PO_4	No activity	
lysozyme/EDTA /diacetyl	23	diacetyl EDTA diacetyl*EDTA	0.4498 0.3115 0.1595	no no no

Table 3.4. Results of combination treatments against *Lactobacillus sakei* grown at 24 °C in APT broth, anaerobically.

Interaction $\alpha = 0.10$	n	Effect	Prob>F	Interaction $\alpha = 0.10$
lysozyme/nisin	33	nisin	0.7635	no
lysozyme/nisin	53	nisin	0.7952	no
/EDTA		EDTA	0.7270	no
		nisin*EDTA	0.8468	no
lysozyme/nisin	54	nisin	0.3713	no
/diacetyl		diacetyl	0.1393	no
		nisin*diacetyl	0.1379	no
lysozyme/nisin	54	nisin	0.6374	no
/tripoly PO_4		tripoly PO_4	0.5276	no
		nisin*tripoly PO_4	0.9215	no
lysozyme/EDTA	12	diacetyl	0.7228	no
/diacetyl		EDTA	0.8829	no
		diacetyl*EDTA	0.7899	no

Table 3.5. Results of combination treatments against *Lactobacillus curvatus* grown at 24 °C in APT broth, anaerobically.

Combination	n	Effect	Prob>F	Interaction $\alpha = 0.10$
lysozyme/nisin	16	nisin	0.4708	no
lysozyme/nisin	58	nisin	0.6421	no
/EDTA		EDTA	0.6173	no
		nisin*EDTA	0.9784	no
lysozyme/nisin	30	nisin	0.1976	no
/diacetyl		diacetyl	0.7232	no
		nisin*diacetyl	0.7235	no
lysozyme/nisin	30	nisin	0.5539	no
/tripoly PO_4		tripoly PO_4	0.6319	no
		nisin*tripoly PO_4	0.9417	no
lysozyme/EDTA	20	diacetyl	lost d.f.	no
/diacetyl		EDTA		
		diacetyl*EDTA		

Table 3.6. Results of combination treatments against *Leuconostoc mesenteroides* grown at 24 °C in APT broth, anaerobically.

Combination	n	Effect	Prob>F	Interaction $\alpha = 0.10$
lysozyme/nisin	25	nisin	0.8740	no
lysozyme/nisin /EDTA	64	nisin	0.7065	no
		EDTA	0.4906	no
		nisin*EDTA	0.7770	no
lysozyme/nisin /diacetyl	39	nisin	0.4799	no
		diacetyl	0.6118	no
		nisin*diacetyl	0.8346	no
lysozyme/nisin /tripoly PO_4	32	nisin	0.6733	no
		tripoly PO_4	0.8326	no
		nisin*tripoly PO_4	0.8362	no
lysozyme/EDTA /diacetyl	---	diacetyl	No activity	
		EDTA		
		diacetyl*EDTA		

Table 3.7. Results of combination treatments against *Listeria monocytogenes* grown at 24 °C in BHI broth, aerobically.

Combination	n	Effect	Prob>F	Interaction $\alpha = 0.10$
lysozyme/nisin	29	nisin	0.1593	no
lysozyme/nisin /EDTA	40	nisin	0.1120	no
		EDTA	0.8509	no
		nisin*EDTA	0.3199	no
lysozyme/nisin /diacetyl	42	nisin	0.1529	no
		diacetyl	0.7403	no
		nisin*diacetyl	0.7430	no
lysozyme/nisin /tripoly PO_4	43	nisin	0.3853	no
		tripoly PO_4	0.1372	no
		nisin*tripoly PO_4	0.1126	no
lysozyme/EDTA /diacetyl	16	diacetyl	0.9793	no
		EDTA	0.9912	no
		diacetyl*EDTA	0.9204	no

Table 3.8. Results of combination treatments against *Pediococcus acidilactici* grown at 24 °C in APT broth, anaerobically.

Combination	n	Effect	Prob>F	Interaction $\alpha = 0.10$
lysozyme/nisin	32	nisin	0.4645	no experimental
lysozyme/nisin /EDTA	64	nisin	0.6372	no
		EDTA	0.9273	no
		nisin*EDTA	0.9872	no
lysozyme/nisin /diacetyl	43	nisin	0.4254	no
		diacetyl	0.5115	no
		nisin*diacetyl	0.7435	no
lysozyme/nisin /tripoly PO_4	40	nisin	0.4777	no
		tripoly PO_4	0.9981	no
		nisin*tripoly PO_4	0.8805	no
lysozyme/EDTA /diacetyl	13	diacetyl	0.2174	no
		EDTA	0.2201	no
		diacetyl*EDTA	0.2177	no

Table 3.9. Results of combination treatments against *Pediococcus pentosaceus* grown at 24 °C in APT broth, anaerobically.

Combination	n	Effect	Prob>F	Interaction $\alpha = 0.10$
lysozyme/nisin	23	nisin	0.3786	no
lysozyme/nisin	55	nisin	0.6355	no
/EDTA		EDTA	0.3278	no
		nisin*EDTA	0.6811	no
lysozyme/nisin	37	nisin	0.8581	no
/diacetyl		diacetyl	0.4965	no
		nisin*diacetyl	0.8925	no
lysozyme/nisin	38	nisin	0.8559	no
/tripoly PO_4		tripoly PO_4	0.4125	no
		nisin*tripoly PO_4	0.7463	no
lysozyme/EDTA	12	diacetyl	0.4266	no
/diacetyl		EDTA	0.4036	no
		diacetyl*EDTA	0.4168	no

Table 3.10. Results of combination treatments against *Salmonella typhimurium* grown at 24 °C in BHI broth, anaerobically.

Combination	n	Effect	Prob>F	Interaction $\alpha = 0.10$
lysozyme/nisin	---	nisin	No activity	
lysozyme/nisin /EDTA	---	nisin EDTA nisin*EDTA	No activity	
lysozyme/nisin /diacetyl	22	nisin diacetyl nisin*diacetyl	0.2079 0.4406 0.2549	no no no
lysozyme/nisin /tripoly PO_4	---	nisin tripoly PO_4 nisin*tripoly PO_4	No activity	
lysozyme/EDTA /diacetyl	15	diacetyl EDTA diacetyl*EDTA	0.7944 0.6086 0.6157	no no no

Table 3.11. Results of combination treatments against *Serratia grimesii* grown at 24 °C in BHI broth, anaerobically.

Combination	n	Effect	Prob>F
lysozyme/nisin	---	nisin	No activity
lysozyme/nisin /EDTA	---	nisin EDTA nisin*EDTA	No activity
lysozyme/nisin /diacetyl	---	nisin diacetyl nisin*diacetyl	No activity
lysozyme/nisin /tripoly PO_4	---	nisin tripoly PO_4 nisin*tripoly PO_4	No activity
lysozyme/EDTA /diacetyl	---	diacetyl EDTA diacetyl*EDTA	No activity

Table 3.12. Results of combination treatments against *Staphylococcus aureus* grown at 24 °C in BHI broth, aerobically.

Combination	n	Effect	Prob>F	Interaction $\alpha = 0.10$	Type of Interaction
lysozyme/nisin	29	nisin	0.5993	no	
lysozyme/nisin /EDTA	58	nisin	0.4889	no	
		EDTA	0.5312	no	
		nisin*EDTA	0.5226	no	
lysozyme/nisin /diacetyl	57	nisin	0.3382	no	
		diacetyl	0.4087	no	
		nisin*diacetyl	0.7901	no	
lysozyme/nisin /tripoly PO_4	42	nisin	0.5934	no	
		tripoly PO_4	0.5232	no	
		nisin*tripoly PO_4	0.5365	no	
lysozyme/EDTA /diacetyl	29	diacetyl	0.5272	no	
		EDTA	0.7259	no	
		diacetyl*EDTA	0.0621	yes	positive

Table 3.13. Results of combination treatments against *Enterococcus faecalis* grown at 24 °C in APT broth, anaerobically.

Combination	n	Effect	Prob>F	Interaction $\alpha = 0.10$
lysozyme/nisin	16	nisin	0.9079	no
lysozyme/nisin /EDTA	40	nisin	0.8662	no
		EDTA	0.6980	no
		nisin*EDTA	0.8153	no
lysozyme/nisin /diacetyl	37	nisin	0.6675	no
		diacetyl	0.3141	no
		nisin*diacetyl	0.6174	no
lysozyme/nisin /tripoly PO_4	29	nisin	0.8960	no
		tripoly PO_4	0.6572	no
		nisin*tripoly PO_4	0.8299	no
lysozyme/EDTA /diacetyl	34	diacetyl	0.2654	no
		EDTA	0.2205	no
		diacetyl*EDTA	0.4529	no

Table 3.14. Results of combination treatments against *Weissella viridescens* grown at 24 °C in APT broth, anaerobically.

Combination	n	Effect	Prob>F	Interaction $\alpha = 0.10$
lysozyme/nisin	12	nisin	0.9718	no
lysozyme/nisin	18	nisin	0.9242	no
/EDTA		EDTA	0.5060	no
		nisin*EDTA	0.5435	no
lysozyme/nisin	26	nisin	0.6499	no
/diacetyl		diacetyl	0.9561	no
		nisin*diacetyl	0.4859	no
lysozyme/nisin	19	nisin	0.5743	no
/tripoly PO_4		tripoly PO_4	0.8736	no
		nisin*tripoly PO_4	0.4999	no
Lysozyme/EDTA	9	diacetyl	0.7102	no
/diacetyl		EDTA	0.6968	no
		diacetyl*EDTA	0.7045	no

4. Interactive inhibition of meat spoilage and pathogenic bacteria by lysozyme, nisin and EDTA in the presence of nitrite and sodium chloride at 24 °C.

4.1. Abstract

A two level, five factor, full factorial experimental design was used to screen for interactions between the antimicrobials, lysozyme (0, 450 mg/L), nisin (0, 450 mg/L), EDTA (0, 900 mg/L), NaCl (0, 0.27 g/L) and $NaNO_2$ (0, 180 mg/L), by F test ($\alpha = 0.01$). Fourteen organisms of concern in the spoilage or safety of cured meat products were grown in broth media. The absorbance at 450 nm relative to inoculated controls was determined after 72 h.

Agents alone reducing growth were: lysozyme against *Brochothrix thermosphacta, Pediococcus acidilactici, Enterococcus faecalis* and *Weissella viridescens*; nisin against all Gram positive organisms tested, including *Lactobacillus sakei* and *Pediococcus pentosaceus*; EDTA against all organisms tested; NaCl or nitrite against *Escherichia coli* O157:H7, *Salmonella typhimurium, Serratia grimesii*, and *Shewanella putrefaciens*; nitrite against *Lactobacillus curvatus, Leuconostoc mesenteroides, Listeria monocytogenes* and *Staphylococcus aureus.*

Interactions were observed between: lysozyme and EDTA (*En. faecalis* and *W. viridescens*); nisin and EDTA (all Gram positive organisms); EDTA and NaCl (*E. coli, S. typhimurium, Ser. grimesii*); EDTA and nitrite (*E. coli, Lb. curvatus, Lc. mesenteroides, L. monocytogenes, S. typhimurium*); nisin and nitrite (*Lc. mesenteroides, L. monocytogenes*); NaCl and nitrite (*S. typhimurium, Sh. putrefaciens*). All the interactions observed reduced

growth.

4.2. Introduction

Lysozyme is an enzyme which possesses activity against the β(1-4) glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine of the bacterial cell wall, resulting in cell lysis (Proctor and Cunningham, 1988). Lysozyme is a 14.6 kDa single peptide protein which is produced by many animals, including man (Proctor and Cunningham, 1988). Applications of lysozyme in foods are currently limited by the high resistance of Gram negative organisms, whose cell wall is protected from interaction with lysozyme by the outer membrane of the cell.

Nisin is a bacteriocin produced by the lactic acid bacterium (LAB) *Lactococcus lactis*. Nisin is most effective as an antimicrobial against LAB and other Gram positive organisms, notably clostridia spp. (Delves-Broughton et al., 1996). Nisin interacts with the cell membrane of sensitive organisms, resulting in leakage of low molecular weight cytoplasmic components and the destruction of the proton motive force (PMF) (Bruno et al., 1992). Two models for nisin activity have been postulated. The "detergent-disruption" model in which the nisin molecule is postulated to disrupt the physical structure of the cell membrane in a manner similar to a detergent. An alternative theory is the "poration complex" model which postulates the formation of pores in the cell membrane by the aggregation of nisin monomers (Montville at al., 1995).

Due to the resistance of Gram negative organisms toward lysozyme and nisin we also

investigated the use of diacetyl as an antimicrobial. Diacetyl has been demonstrated to be an effective antimicrobial against a wide range of Gram negative and Gram positive bacteria, though LAB are generally resistant (Jay, 1982a,b: Jay et al., 1983; Jay and Rivers, 1984). Kang and Fung (1999) have demonstrated that addition of 300 mg/Kg of diacetyl to salami fermentation reduced *E. coli* and *S. typhimurium* numbers by 1 Log CFU/g over a 24 h. incubation, without reducing acid production by *P. acidilactici*. The resistance of LAB to diacetyl was seen as potentially adventitious. In vacuum packaged cured meat products, LAB extend shelf life and there was some concern that the use of an antimicrobial system which was effective against LAB, but ineffective against Gram negative organisms could result in the latter being in a position to colonize products without competition from the normally dominant LAB. The mechanism of diacetyl antimicrobial action is unknown though Jay et al. (1983) suggested that it may result from inhibition of arginine metabolism.

A body of work has been produced regarding the use of chelators, with lysozyme (Hughey and Johnson, 1987; Ellison and Giehl, 1991; Payne et al., 1994) or nisin (Stevens et al., 1991, 1992; Cutter and Siragusa, 1995a,b). The antimicrobial activity of chelators, such as tripolyphosphate and ethylenediamine tetraacetate (EDTA), is by limiting the availability of cations and complexing the divalent cations which act as salt bridges between membrane macromolecules, such as lipopolysaccharides (for reviews see, Proctor and Cunningham, 1988; Delves-Broughton et al., 1996; Shelef and Seiter, 1993).

In this study we conducted evaluations of the activity of lysozyme in combination with nisin, diacetyl and the chelator EDTA, having hypothesized that the different target activities

of the three agents may result in an increased bacteriostatic or bactericidal activity due to antimicrobial interactions. The experiments were conducted in nutrient broth to evaluate the potential effectiveness of lysozyme, used alone and in combination with other agents, as an antimicrobial for use in cured meat products to enhance shelf life and product safety.

The minimum inhibitory concentration (MIC) of the individual antimicrobial agents were determined. On the basis of the MIC values two agents, nisin and EDTA were selected for investigation to determine if any interactions with lysozyme or each other occurred that might result in an increased inhibition of microbial growth. Since the intention of these experiments was to develop an antimicrobial treatment for use in cured meat products, screening tests for interactions with nitrite and NaCl were also conducted.

The inclusion of nitrite and NaCl in tests for possible interactions was also conducted as it has been reported that high ionic concentrations have a negative effect on the activity of lysozyme (Davies et al., 1969). Additionally, the presence of numerous freely available ions in complex media can also be expected to reduce the membrane disruption effect of chelators. Nitrite and NaCl are two common ingredients of cured meat products which are widely held to contribute to the restriction of the growth of some bacterial species and these also serve as an additional source of ions in processed meats. Thus it was important to determine whether the activity of lysozyme, nisin and EDTA was changed in their presence.

To screen for interactions between the five agents a two level, full factorial, experimental design was used. The concentrations of the agents used were; lysozyme (0, 450

mg/L), nisin (0, 450 mg/L), EDTA (0, 900 mg/L), sodium chloride (0, 0.27 g/L), and sodium nitrite (0, 180 mg/L). The levels of lysozyme, nisin and EDTA were chosen on the basis of effective MIC concentrations. The concentrations of NaCl and nitrite were selected as representing an average range of concentrations found in cured meat products (≤200 mg/L nitrite is permitted in cured meats, except bacon, in Canada).

The organisms tested were: *Brochothrix (B.) thermosphacta*, *Escherichia (E.) coli* O157:H7, *Lactobacillus (Lb.) sake*, *Lactobacillus (Lb.) curvatus*, *Leuconostoc (Lc.) mesenteroides*, *Listeria (L.) monocytogenes*, *Pediococcus (P.) acidilactici*, *Pediococcus (P.) pentosaceus*, *Salmonella (S.) typhimurium*, *Serratia (Ser.) grimesii*, *Staphylococcus (St.) aureus*, *Shewanella (Sh.) putrefaciens*, *Enterococcus (En.) faecalis*, and *Weissella (W.) viridescens*. These organisms were selected as organisms representative of spoilage or safety concern, which may be found comprising the flora of cured meat products. The tests were conducted in BHI or APT media, as appropriate, buffered to pH 6.0 with morpholinoethane sulphonic acid (MES) (Bunic et al., 1995) to mimic the pH conditions found in cured meat products.

4.3. Materials and Methods

4.3.1. Cultures Used, Source and Incubation Conditions

B. thermosphacta B2 was from Dr G. Greer Agriculture and Agrifood Canada (AAFC) Research Station, Lacombe, AB; BHI; aerobic. *E. coli* O157:H7 strain E318 was a human clinical isolate provided by Dr. R. Johnson, Health Canada, Guelph , ON; BHI; aerobic. *Lb. sake* #7, *Lb. curvatus* #15 and *Lc. mesenteroides* #11, were isolated from spoiled

cured meats (Holley et al., 1996); APT; anaerobic. *Listeria monocytogenes*, University of Manitoba, Department of Food Science culture collection; BHI; aerobic. *P. acidilactici* 122P and *P. pentosaceus* 116P, were isolated from commercial meat starter cultures; APT; anaerobic. *S. typhimurium* #98 was from the AAFC collection, Guelph ON; BHI; anaerobic. *Ser. grimesii* S12 was isolated from cooked roast beef, AAFC collection, Summerland, BC; BHI; anaerobic. *Sh. putrefaciens*, was from the ATCC as #8071; BHI, aerobic. *St. aureus* # 427 was from the AAFC collection, Guelph ON; BHI; aerobic. *En. faecalis* #28 (ATCC #7080), was from the AAFC collection, Guelph, ON; BHI; anaerobic. *W. viridescens* # 13 (ATCC # 12706), was from the AAFC collection, Guelph ON; APT; anaerobic.

Cultures were maintained monthly on agar slants at 4 °C and as frozen glycerol stocks. Cultures were streak-plated once a week and cultures for experiments were inoculated into media from a single colony and incubated overnight under the appropriate atmospheric conditions. All cultures were maintained in either APT or BHI media, except for *Lb. sake* #7 which was maintained on M17 agar but grown in APT. Incubations were conducted at room temperature, (24 °C).

4.3.2. Materials

Lysozyme HCl (lot# A7333-F) and nisin (2.5 g/Kg w/w Chrisin, lot# 17127) were provided by Canadian Inovatech, Abbotsford, BC. Morpholinoethane sulfonic acid, MES (2-4-morpholino-ethane sulfonic acid), EDTA (disodium ethylenediamine tetraacetate) and tripolyphosphate (sodium phosphate tripoly) were obtained from Fisher Scientific, Toronto, ON. Diacetyl (2,3-butadione) was from Aldrich Chemicals, Oakville, ON. The APT broth

(all purpose tween broth) was from BBL, Becton-Dickinson, Franklin Lakes, NJ . BHI broth (brain heart infusion broth) and granulated agar, were from DIFCO, Detroit, Michigan. The M17 broth was from Oxoid, Nepean, ON. GasPak Jars (BBL, Becton-Dickinson) were made anaerobic using the GasPak Plus Anaerobic System with palladium catalyst. An anaerobic incubator (National Appliance Co., Portland, Oregon) flushed 3 times with CO_2 was also used for anaerobic incubations. Growth was monitored in 96 well polypropylene micro-titre plates (Corning-Costar, Corning Incorporated, Acton, MA), using a Titretek Multiskan MCC/340 Mk II type 347 spectrophotometer (Flow Laboratories International SA, Switzerland), at a wavelength of 450 nm.

4.3.3. Determination of MIC

The protocol described here was adapted from that of Maclean et al. (1997a,b). The media used in these experiments were either ATP or BHI broths, and both were prepared with 10 g/L MES buffer, adjusted to pH 6.0 with 0.1 mol/L HCl or NaOH.

Each well of a sterile 96 micro-titre plate was filled with 100 µL of APT or BHI broth. All media containing the agents to be tested were prepared freshly each day. Lysozyme, nisin and diacetyl were prepared as 1 mg/mL solutions, dissolved in previously sterilized media broth. Standard stock solutions of EDTA and tripolyphosphate were prepared in deionized water and filter sterilized when added to previously autoclaved broth media, to achieve the desired final concentration.

The first column of wells in each micro-titre plate received 100 µL of broth media

containing the agent to be tested. After mixing, 100 μL was transferred to the second column of wells. The process of serial dilution was repeated until the last column, #12, was reached and it was left without the agent.

Each bacterium tested was inoculated from a single colony into 5 mL of APT or BHI broth in a test tube and incubated overnight. Incubation was at room temperature under aerobic or anaerobic conditions, as appropriate. Serial 1/100 dilutions (50 μL in 5 mL) of overnight culture were done in media broth to 10^{-6}. The inoculum was enumerated by surface plating the 10^{-6} dilution on to solidified agar plates which were incubated for two d. at 24 °C and then counted. Each well in the micro-titre plates, except four in column #12, was inoculated with 10 μL of a 10^{-4} dilution of the culture to be tested. Four wells (inoculated, no agent), in column #12, served as a positive control for growth and four (uninoculated, no agent) as a negative control for growth.

The inoculated micro-titre plates were then incubated for 60 h. at 24 °C (room temperature), under the appropriate atmospheric conditions. The wells were scored visually as growth or no growth. The concentration in the lowest serial dilution of the agent at which growth did not occur was recorded as the minimal inhibitory concentration (MIC). Experiments were repeated four times.

4.3.4. Interaction Screening

The media also used in these experiments was ATP or BHI broths and both were prepared with 10 g/L MES buffer, adjusted to pH 6.0 with 0.1 mol/L HCl or NaOH.

The experiments were conducted in 96 well polypropylene micro-titre plates, with four plates being used for each organism. In columns 1-10 of each plate, appropriate volumes of the six inhibitor test agent solutions and growth media were added to each well to produce all 32 combinations of the five agents at minimal or maximal concentration, with a final volume of 100 μL. With a 10 μL inoculum the final concentrations of the 5 agents were: lysozyme, 0/450 mg/L; nisin, 0/450 mg/L; EDTA, 0/900 mg/L; $NaNO_2$ 0/180; NaCl 0/0.27g/L. Column 11 contained 100 μL media without added test agents and was inoculated as a positive control. Column 12 contained 110 μL of media and was not inoculated so as to serve as a negative control.

Each strain to be tested was inoculated from a single colony into 5 mL of APT or BHI broth in a test tube and incubated overnight. Incubation was at 24 °C under aerobic or anaerobic conditions, as appropriate. Serial 1/100 dilutions (50 μL in 5 mL) of overnight culture were made in media broth to 10^{-6}. The inoculum was enumerated by plating the 10^{-6} dilution on to agar media plates which were incubated for 3 d. and then counted. Each well in the micro-titre plates was inoculated with 10 μL of a 10^{-4} dilution of the culture to be tested, except for the wells of column 12. The inoculated plates were then incubated for 3 d. at 24 °C.

After incubation had been completed, the absorbance at 450 nm was measured using a Titretek Multiscanner. By the deduction of the average of the negative control wells on each plate (110 μL of uninoculated media) the net absorbance of the wells for each treatment was determined. These values were used to calculate the average relative absorbance of treatment

wells compared to the positive controls (110 μL of inoculated media). The values for relative absorbance were then entered into the statistical model for analysis. Values for relative absorbance were calculated from 10 replicates.

4.3.5. Statistical Analysis

These experiments were analyzed using a two level test to screen for paired interactions among five antimicrobial agents (lysozyme, nisin, EDTA, NaCl and nitrite). The experiment was designed with 32 treatments (or combinations of the agents) as this was the minimum number that would allow resolution of two way interactions between agents without confounding effects between agents. The pattern of treatments is shown in Table 4.1.

The values for relative absorbance were determined for each treatment and entered into the experimental design as the response. The JMP program (SAS Institute) was used to evaluate the probability that individual agents significantly affected the response (relative absorbance) by conducting an F test, the results of which are described by the value Prob>F. Prob>F is the "probability of being wrong if you declare an effect to be non null" (Sall and Lehman, 1996). This can be interpreted as the probability that an agent or combination of agents does not affect the relative absorbance of the inoculated media after incubation. It is assumed that the relative absorption of inoculated media is related to the growth of the test organism.

The apparent effect of an agent or its interaction with a second agent on the relative absorbance was determined using an alpha value of 0.01. In other words, it was determined that the agent or interaction had no effect on the relative absorbance when there was a greater

than 0.01 probability of being wrong if the null hypothesis (no effect) were rejected. The effect on growth was characterized as negative (decreasing absorption or growth) or positive (increasing absorption or growth) by the direction of the slope of the response predicted by the statistically generated model. A confidence level of P=0.01 was chosen, as the use of a high maximal level (ie. test concentration) and zero minimal level should result in a highly significant effect upon the response.

4.4. Results

The MICs determined for inhibition of growth by lysozyme, nisin, EDTA, tripolyphosphate and diacetyl are reported in Table 3.1 as mg/L. Where no inhibition of growth was observed, the highest concentration of agent tested was reported. Of the organisms tested, only *B. thermosphacta* was inhibited by concentrations of lysozyme of 500 mg/L or less. Nisin was effective against all of the Gram positive organisms tested, at varied concentrations. Nisin was ineffective against all of the Gram negative organisms at the concentrations tested. EDTA was inhibitory against all of the organisms tested except *S. typhimurium* and *Ser. grimesii*. Diacetyl was inhibitory towards *S. typhimurium* and *E. coli* O157:H7 at ≥250 mg/L. Tripolyphosphate was ineffective as an antimicrobial agent at the concentrations tested.

Interaction screening experiments were not conducted using tripolyphosphate due to its poor antimicrobial activity. Diacetyl was rejected for further investigation as at the concentrations required for inhibition caused sensory changes (buttery odor) that made it unsuitable for application to meat products.

The results of the two level, five factor study are presented in Tables 4.2 to 4.4. Lysozyme alone was observed to be effective against *B. thermosphacta, P. acidilactici, En. faecalis* and *W. viridescens*. The results also showed growth inhibitory effects by both NaCl and nitrite on *E. coli* O157:H7, *S. typhimurium, Ser. grimesii*, and *Sh. putrefaciens*. The following organisms were sensitive to the presence of nitrite, though not NaCl: *Lb. curvatus, Lc.mesenteroides, L. monocytogenes*, and *St. aureus*.

Statistical analysis of the results indicated that a group of interactions occurred which were all negative (reduced growth). For both *En. faecalis* and *W. viridescens* an interaction was observed between lysozyme and EDTA. An interaction was observed between nisin and EDTA for all of the Gram positive organisms tested. Various different patterns of interactions were observed among the organisms tested including: EDTA and NaCl (*E. coli, S. typhimurium, Ser. grimesii*); EDTA and nitrite (*E. coli, Lb. curvatus, Lc. mesenteroides, L. monocytogenes, S. typhimurium*); nisin and nitrite (*Lc. mesenteroides, L. monocytogenes*); and NaCl and nitrite (*S. typhimurium, Sh. putrefaciens*). No antagonistic interactions were observed between the factors tested.

4.5. Discussion

In this study no increase in antimicrobial activity was observed when nisin was used with EDTA or when lysozyme was used with EDTA against any of the Gram negative organisms tested. In contrast, a number of authors have reported observation of increased antimicrobial activity by lysozyme (Shively and Hartsell, 1964a,b; Hughey and Johnson 1987, Ellison and Giehl, 1991, Payne et al., 1994, Park, 1997) or nisin (Stevens et al., 1991, 1992;

Cutter and Siragusa, 1995b; Carneiro De Melo et al., 1998) against Gram negative and Gram positive organisms in the presence of membrane disrupting agents or sub-lethal injury (Kalachyanand, 1992). The majority of these reports were based upon observation of organisms suspended in a buffer, rather than when growing in nutrient media (Shively and Hartsell, 1964a,b; Ellison and Giehl, 1991, Stevens et al., 1991, 1992; Cutter and Siragusa, 1995b; Kalachyanand, 1992). Although in one study (Carneiro De Melo et al., 1998), the authors used distilled water as a suspension medium. Where studies have been conducted in media capable of supporting growth, no activity against Gram negative organisms has been reported, except where unrealistically high levels of agents for food applications have been used. Park (1997) observed lysozyme activity against *E. coli* in nutrient broth and vegetable juice at levels of 0.1 to 0.4 g/L. Cutter and Siragusa (1995b) reported antimicrobial activity of nisin plus chelators against *E. coli* and *Salmonella* in buffer, but reported no significant effect upon the same organisms when trials were conducted on beef (1995a).

Since food products provide a nutrient rich environment, tests of antimicrobials under conditions of cell starvation would appear to be of limited value in evaluating them for application in food products. For this reason our experiments were designed to examine for interactions between the antimicrobial agents of interest under non-nutrient restricted conditions. The failure to observe interaction between lysozyme or nisin with EDTA in this study may indicate that the observed interaction between these antimicrobials in buffer systems are a consequence of cell starvation which prevents cell repair.

The two level, five factor experimental design used in this study allowed identification

of each factor which may contribute significantly to the response and whether any two way interaction between factors occurred. This approach provided certain advantages over the checker-board method (Maclean et al., 1997a,b) for preliminary evaluation of antimicrobials, prior to studies in food systems. This method was as rapid and inexpensive as the checker-board method but can provide considerably more information by allowing observation of responses in systems containing more than two antimicrobial agents. Additionally, evaluation of results based on a variable response rather than the growth/no-growth scoring of the checker-board method allowed the determination of effects on growth in cases where the growth rate was slowed as well as where growth was inhibited.

The method presented here does possess some limitations. Interactions between factors can only be detected where the effect of one factor alone does not exceed the maximum response. Further, if a single agent should inhibit growth below the detectable level then any further inhibition by other factors will not allow detection of the initial inhibitory event and will not contribute to the statistical model. In addition, although the relative absorbance of the media can be related to the bacterial population level due to increasing turbidity, bacterial populations below 10^5 CFU/mL have little or no effect on turbidity.

The results of these experiment indicated a complex pattern of interactions between pairs of agents, which differed between different organisms. However, there were no interactions observed between lysozyme, nisin and EDTA that enhanced their effectiveness against any of the Gram negative organisms. In contrast, several interactions were observed between lysozyme or nisin and EDTA, that resulted in enhanced activity against several Gram

positive organisms.

There was no evidence that the presence of nitrite and NaCl prevented the antimicrobial activity of lysozyme, nisin and EDTA against any of the organisms used in this study. In fact, several interactions which enhanced inhibition were observed between nisin or EDTA and nitrite or NaCl. These interactions included enhancement of activity against Gram negative organisms, such as EDTA and NaCl (*E. coli, S. typhimurium, Ser. grimesii*); EDTA and nitrite (*E. coli, S. typhimurium*); and NaCl and nitrite (*S. typhimurium, Sh. putrefaciens*).

These results indicate that the activity of lysozyme and nisin was enhanced against the tested Gram positive organisms in the presence of EDTA under conditions in which growth is not restricted by starvation. There was no protective effect of nitrite or NaCl upon the inhibitory action of lysozyme, nisin and EDTA. Further, the results indicated that the addition of lysozyme, nisin and EDTA to cured meat products containing NaCl and nitrite should result in a wider spectrum of antimicrobial activity.

The results of these experiments suggested that the application of a lysozyme, nisin and EDTA antimicrobial system for use in cured meat products warrants further investigation in the form of trials utilizing commercial product held at slightly abusive but moderately restrictive temperature.

Table 4.1. Pattern of treatments used for the 2 level[1], 5 factor[2] experimental design against 14 different organisms.

	Lysozyme 450 mg/L	Nisin 450 mg/L	EDTA 900 mg/L	NaCl 0.27 g/L	$NaNO_2$ 180 mg/L
Treatment design group intervals	16	8	4	2	alternate
Tube number containing agent	16-32	9-16 25-32	5-8 3-16 21-24 29-32	3-4 7-8 11-12 15-16 19-20 23-24 27-28 31-32	all even tubes

1. Tubes identified in the table contained the test agent concentrations noted. The remaining 16 tubes did not contain the agent.

2. Each agent was tested individually and with 1-4 other agents against the 14 organisms. All combinations were tested . Treatment 1 contained no agents, treatment 32 contained all agents. The experiment was repeated 10 times.

Table 4.2. Significance[1] of two agent inhibitory interactions against several Gram positive bacteria studied in 5 factor, 2 level tests.

Effect	*B. thermosphacta*	*W. viridescens*	*Lb. sakei*	*Lb. curvatus*	*Lc. mesenteroides*
lysozyme	0.0049[a]	0.0070[a]	0.2122	0.1594	0.7718
nisin	0.0003[a]	<0.0001[a]	<0.0001[a]	<0.0001[a]	<0.0001[a]
lysozyme*nisin	0.0949	0.0137	0.0923	0.2445	0.9386
EDTA	0.0004[a]	<0.0001[a]	<0.0001[a]	<0.0001[a]	<0.0001[a]
lysozyme*EDTA	0.0957	0.0128	0.0698	0.2251	0.5303
nisin*EDTA	0.0035[a]	<0.0001[a]	<0.0001[a]	<0.0001[a]	<0.0001[a]
NaCl	0.6873	0.7237	0.0833	0.2326	0.5434
lysozyme*NaCl	0.3060	0.4386	0.1305	0.8052	0.6660
nisin*NaCl	0.6435	0.7390	0.6723	0.2684	0.5170
EDTA*NaCl	0.5884	0.7806	0.6588	0.4510	0.9362
nitrite	0.0109	0.0183	0.0196	0.0027[a]	<0.0001[a]
lysozyme*nitrite	0.3662	0.6233	0.4331	0.8209	0.5613
nisin*nitrite	0.0496	0.0293	0.1327	0.0309	0.0004[a]
EDTA*nitrite	0.0588	0.0273	0.1827	0.0058[a]	0.0035[a]
NaCl*nitrite	0.7533	0.9801	0.3915	0.9259	0.8058

1. Values tabulated are Prob>F. These represent the probability of being wrong if an effect is declared non-null (inhibitory). The effect of an agent or its interaction with a second agent upon absorbance was considered significant using an alpha (a) value of 0.01. In all instances of statistical significance (denoted by superscript "a"), the interaction had a negative effect on bacterial growth.

Table 4.3. Significance[1] of two agent inhibitory interactions against several Gram positive bacteria studied in 5 factor, 2 level tests.

Effect	*En. faecalis*	*P. pentosaceus*	*P. acidilactici*	*St. aureus*	*L. monocytogenes*
lysozyme	0.0022[a]	0.0790	0.0038[a]	0.7608	0.0544
nisin	<0.0001[a]	<0.0001[a]	<0.0001[a]	0.0004[a]	<0.0001[a]
lysozyme*nisin	0.3207	0.0321	0.1231	0.8561	0.1758
EDTA	<0.0001[a]	<0.0001[a]	<0.0001[a]	0.0004[a]	<0.0001[a]
lysozyme*EDTA	0.0044[a]	0.0479	0.0919	0.6563	0.2220
nisin*EDTA	<0.0001[a]	<0.0001[a]	<0.0001[a]	0.0109	0.0006[a]
NaCl	0.2079	0.6658	0.9644	0.1095	0.3041
lysozyme*NaCl	0.8906	0.1463	0.0835	0.5417	0.7413
nisin*NaCl	0.7709	0.6232	0.1231	0.2992	0.5362
EDTA*NaCl	0.3687	0.4476	0.6874	0.3980	0.5913
nitrite	0.2293	0.5280	0.5266	0.0005[a]	0.0002[a]
lysozyme*nitrite	0.5111	0.3399	0.1512	0.5340	0.5281
nisin*nitrite	0.5906	0.0920	0.8620	0.0109	0.0036[a]
EDTA*nitrite	0.2128	0.1493	0.6983	0.0132	0.0041[a]
NaCl*nitrite	0.4727	0.3341	0.4653	0.2711	0.9169

1. Values tabulated are Prob>F. These represent the probability of being wrong if an effect is declared non-null (inhibitory). The effect of an agent or its interaction with a second agent upon absorbance was considered significant using an alpha (a) value of 0.01. In all instances of statistical significance (denoted by superscript "a"), the interaction had a negative effect on bacterial growth.

Table 4.4. Significance[1] of two agent inhibitory interactions against several Gram negative bacteria studied in 5 factor, 2 level tests.

Effect	*S. typhimurium*	*E. coli*	*Ser. grimesii*	*Sh. putrefaciens*
lysozyme	0.3189	0.0153	0.1556	0.1784
nisin	0.5993	0.6437	0.6618	0.2516
lysozyme*nisin	0.0748	0.1529	0.3384	0.7111
EDTA	<0.0001[a]	<0.0001[a]	<0.0001[a]	0.0003[a]
lysozyme*EDTA	0.5999	0.0892	0.3206	0.3501
nisin*EDTA	0.8898	0.7299	0.4322	0.4261
NaCl	<0.0001[a]	<0.0001[a]	0.0018[a]	0.0003[a]
lysozyme*NaCl	0.5224	0.5352	0.9663	0.5006
nisin*NaCl	0.8079	0.7937	0.3522	0.4651
EDTA*NaCl	0.0049[a]	<0.0001[a]	0.0029[a]	0.0138
nitrite	<0.0001[a]	<0.0001[a]	<0.0001[a]	0.0004[a]
lysozyme*nitrite	0.4991	0.3633	0.5022	0.3490
nisin*nitrite	0.5303	0.8590	0.7857	0.5420
EDTA*nitrite	<0.0001[a]	<0.0001[a]	0.1146	0.0114
NaCl*nitrite	<0.0001[a]	0.8488	0.5689	0.0076[a]

1. Values tabulated are Prob>F. These represent the probability of being wrong if an effect is declared non-null (inhibitory). The effect of an agent or its interaction with a second agent upon absorbance was considered significant using an alpha (a) value of 0.01. In all instances of statistical significance (denoted by superscript "a"), the interaction had a negative effect on bacterial growth.

5. Inhibition of bacterial growth on ham and bologna by lysozyme, nisin and EDTA

5.1. Abstract

Ham and bologna sausages were prepared with or without addition of 500 $mg.kg^{-1}$ lysozyme:nisin::1:3, and 500 $mg.kg^{-1}$ EDTA. Sausages were inoculated with one of; *Brochothrix thermosphacta*, *Escherichia coli* O157:H7, *Lactobacillus sakei*, *Lactobacillus curvatus*, *Leuconostoc mesenteroides*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Serratia grimesii* or *Shewanella putrefaciens*, vacuum packed and stored for 4 weeks at 8 °C. Plate counts were made on selective and nonselective media.

Inhibitor treatment reduced initial populations of *B. thermosphacta* and *Lc. mesenteroides* on both meats. Treatment of ham and bologna prevented growth of *B. thermosphacta*, to week 4. Treatment reduced growth of *Lb. curvatus* on ham and bologna, to week 3. Treatment of bologna reduced growth of *Lc. mesenteroides* and *L. monocytogenes* for 2 weeks. Treatment of ham reduced growth of *E. coli* O157:H7 for 4 weeks. On treated ham the growth of *S. typhimurium* was increased from week 3. No difference was observed between control and treatment samples with other organisms.

5.2. Introduction

Lysozyme is a 14.6 kDa single peptide protein (Proctor and Cunningham, 1988), which possesses enzymatic activity against the β(1-4) glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine found in peptidoglycan. Peptidoglycan is the major component of the cell wall of both Gram positive and Gram negative bacteria.

Hydrolysis of the cell wall by lysozyme can damage the structural integrity of the cell wall and result in the lysis of bacterial cells. Lysozyme is of interest for use in food systems as it is a naturally occurring enzyme that is produced by many animals, including man, and has activity against a cellular structure specific to bacteria (Proctor and Cunningham, 1988).

The bacteriocin, nisin, has been used as an antimicrobial in foods since the 1960's (for a review see Delves-Broughton, Blackburn, Evans & Hugenholtz, 1996). The activity of nisin, like lysozyme, is specific for bacterial cells. Nisin is produced by the lactic acid bacterium (LAB) *Lactococcus lactis*. Nisin is most effective as an antimicrobial against LAB and other Gram positive organisms, notably clostridia species (Delves-Broughton et al., 1996). The mechanism of nisin activity has been shown to involve alteration of the cell membrane of sensitive organisms resulting in leakage of low molecular weight cytoplasmic components and destruction of the proton motive force (PMF) (Bruno, Kaiser & Montville, 1992). Two models for nisin activity have been postulated. The "detergent-disruption" model is one in which the nisin molecule is thought to disrupt the physical structure of the cell membrane in a manner similar to detergents. An alternative theory is the "poration complex" model which postulates the formation of an oligomeric pore in the cell membrane by the aggregation of nisin monomers to yield cytoplasmic leakage (Montville et al., 1995).

It has been recognized since the 1960's that susceptibility of Gram negative organisms to lysis by lysozyme can be increased by the use of membrane disrupting agents, such as detergents and chelators (Shively and Hartsell, 1964a, 1964b). Ethylenediaminetetraacetate (EDTA), a chelator, can have an antimicrobial effect by limiting the availability of cations and

can act to destabilize the cell membranes of bacteria by complexing divalent cations which act as salt bridges between membrane macromolecules, such as lipopolysaccharrides (Vaara, 1992; Shelef and Seiter, 1993).

Previous experiments in all purpose tween and brain heart infusion broths have indicated that a combination treatment of lysozyme, nisin and EDTA may be effective in controlling the growth of organisms of concern in the spoilage and safety of cured meat products (Chapter 4).

In this series of experiments, commercially formulated ham or bologna batter was used to prepare sausages with 500 $mg.kg^{-1}$ lysozyme:nisin::1:3 plus 500 $mg.kg^{-1}$ EDTA (treatment), or without any inhibitors (control). The products were cooked, cut into 14 mm thick coins and then inoculated with one of 9 organisms: *Brochothrix (B.) thermosphacta*, *Escherichia (E.) coli* O157:H7, *Lactobacillus (Lb.) sakei*, *Lactobacillus (Lb.) curvatus*, *Leuconostoc (Lc.) mesenteroides*, *Listeria (L.) monocytogenes*, *Salmonella (S.) typhimurium*, *Serratia (Ser.) grimesii*, or *Shewanella (Sh.) putrefaciens*. The products were then vacuum packed and incubated at 8 °C. The microbial populations on the meats were monitored by plating onto selective and non-selective media, to determine whether there was a difference between the population levels on control and treated samples.

Studies have been published by a number of authors on the use of lysozyme or nisin as antimicrobials in wide variety of food products (for reviews see, Delves-Broughton, et al., 1996, and Proctor and Cunningham, 1988). Studies on the use of lysozyme or nisin as

antimicrobials for use in meat products have been primarily concerned with surface treatments applied to fresh meat products (Cutter and Siragusa, 1995a and 1997, Sheffet, Sheldon & Klaenhammer, 1995, Murray and Richard 1997). Application of antimicrobial treatments to cured meat products is an area that has received little attention. However, cured meat products may be an excellent system in which to use lysozyme, nisin and EDTA combination treatments, since the presence of other growth restrictive chemicals and conditions, such as nitrite and NaCl (Chapter 4) may increase the effectiveness of antimicrobial treatment against spoilage flora and pathogens.

5.3. Materials and Methods

5.3.1. Cultures Used, Incubation Conditions and Selective Media

B. thermosphacta B2 was from Dr. G. Greer, Agriculture and Agrifood Canada (AAFC) Research Station, Lacombe, AB (brain heart infusion broth, BHI, aerobic). *E. coli* O157:H7 E318 was a human clinical isolate provided by Dr. R Johnson, Health Canada, Guelph, ON, resistant to 30 ppm nalidixic acid (BHI, aerobic). *Lb. sakei* #7, *Lb. curvatus* #15, *Lc. mesenteroides* #11, were isolated from spoiled cured meats (Holley et al., 1996) (all purpose tween broth, APT, anaerobic). *L. monocytogenes*, Univ. of Manitoba, Department of Food Science culture collection (BHI, aerobic). *S. typhimurium* #98; AAFC collection, Guelph ON (BHI, anaerobic). *Ser. grimesii* S12, was isolated from cooked roast beef, AAFC collection, Summerland, BC (BHI, anaerobic). *Sh. putrefaciens*, ATCC #8071, (BHI, aerobic).

Cultures were maintained monthly on agar slants at 4 °C and as frozen glycerol stocks.

Cultures were streak-plated once a week and cultures for experiments were inoculated into media from a single colony and incubated overnight under the appropriate atmospheric conditions. All cultures were maintained in either APT or BHI media, except for *Lb. sakei* #7 which was maintained on M17 agar, but grown in APT broth . The MI7 medium was used for the maintenance of *Lb. sakei* since the glycerol phosphate it contains prevented rapid reduction in pH.

Organisms were recovered from inoculated meat samples using the following selective media: *B. thermosphacta*, aerobic, streptomycin thallous acetate actidione agar (STAA); *E. coli* O157:H7, aerobic, BHI agar with 30 ppm nalidixic acid (BHI+nal); *Lb. curvatus*, *Lb. sakei* and *Lc. mesenteroides*, anaerobic, deMan, Rogosa and Sharpe agar (MRS); *L. monocytogenes*, aerobic, modified oxford medium agar (MOX); *S. typhimurium*, anaerobic, brilliant green agar with sulfadiazine (BGS); *Ser. grimesii*, aerobic, violet red bile glucose agar (VRBG); *Sh. putrefaciens*, aerobic, peptone iron agar (PI). In all cases colonies on selective media were enumerated after 48 h incubation at 24 °C.

5.3.2. Materials

Commercially prepared chopped ham mix (12.5% meat protein, pork, water, salt, sugar, dextrose, sodium phosphate, carrageenan, sodium erythorbate, sodium nitrite) and bologna emulsion (pork, mechanically separated turkey, chicken or pork; beef or beef byproducts, water, wheat flour, potato starch, salt dextrose, spices, sodium erythorbate, sodium nitrite, and smoke) were provided by Maple Leaf Meats, Winnipeg, MB. Sausage casings (hog intestine, 30 mm diameter) were from Canada Compound Western, Winnipeg,

MB. Sausage casings were stuffed with meat emulsions using a 9L capacity mechanical sausage stuffer from F. Dick Gmbh, Germany. Low oxygen permeable polyvinylediene chloride (PVDC) plastic bags were kindly provided by Winpak, Winnipeg, MB (25 x 35 cm, Deli #1). Lysozyme:nisin::1:3 (lot# 9312-26, 23.75 % (w/w) pure lysozyme, 1.69 % (w/w) pure nisin) was premixed and provided by Canadian Inovatech, Abbotsford, BC. EDTA was from Fisher Scientific, Toronto, ON. APT broth, BGS and SMA (standard methods agar) were from BBL, Becton-Dickinson, Franklin Lakes, NJ. BHI broth, granulated agar, MRS broth (Lactobacillus MRS broth), violet red bile bile glucose agar, peptone iron agar, proteose peptone No. 3, oxford medium base, modified oxford antimicrobic supplement (colistin sulfate 10 $mg.L^{-1}$, moxalactam 20 $mg.L^{-1}$) for MOX, and yeast extract were from Difco, Becton-Dickinson. M17 agar was supplied by Oxoid Ltd, Basingstoke, U.K. Streptomycin sulphate, cycloheximide and thallous acetate were from Sigma-Aldrich, Oakville, ON. An Autoplater 4000 equipped with a CASBA-4 automated counting system was used for surface plating on pre-poured agar media and enumeration (Spiral Biotech, Inc., Bethesda, MD). The anaerobic incubator (model 3640-6) was from National Appliance Co., Portland, OR, and anaerobic conditions were created by flushing twice with 30% CO_2/ 70 % N_2.

5.3.3. Methods

The ham and bologna sausages used in these experiments were each prepared from a single batch of chopped ham mix or bologna emulsion. The raw meat batters were obtained from the manufacturer on the day of production and stored at -20 °C until the sausages were to be made.

The meat batters were thawed overnight at 4 °C and then divided into two equal lots. One lot remained untreated and was used to prepare control samples. The treated samples were prepared by the addition of: 500 $mg.kg^{-1}$ of lysozyme:nisin::1:3 and 500 $mg.kg^{-1}$ of EDTA, followed by mechanical mixing to insure homogeneity. Using a 9L capacity hand-cranked sausage stuffer, treated and control sausages of ham and bologna mix were prepared in the natural casings. The casings were originally salted, but were soaked overnight in deionized water at 4 °C to remove the salt. After stuffing to 12 cm lengths, the sausages were clipped with a metal ring, then cooked to an internal temperature of 69 °C, packaged and stored at -20 °C until inoculated. The heating of the sausages was conducted in a jacketed steam kettle for 3 intervals of 20 minutes at temperatures of 52.8 °C, 63.9 °C, and 75 °C. The sausages were then vacuum packed in heat-sealed O_2 barrier (PVDC) plastic bags and stored at -20 °C until required for inoculation. The pH of the cooked sausage was determined by suspending 5 g samples in 45 mL of distilled water.

A 50 mL culture of the organism to be tested was prepared in BHI or APT broth, as appropriate, and incubated aerobically or anaerobically for 48 h at room temperature. A dipping bath for inoculation of the control and inhibititor- treated samples with each test organism was prepared by diluting 1 mL of culture in 1L of 0.1% peptone, resulting in an initial population of approximately 10^4 $CFU.cm^{-2}$ on the meats.

Ham and bologna, control (no inhibitor) and treated sausages were thawed overnight at 4 °C and then cut on a sterile board using a flame-sterilized knife into 14 mm "coins". This produced portions of meat 29-31 mm in diameter and 14 mm on a side, with a surface area

(maximum) of 28.73 cm^2 and weighing 10 g ♠ 1 g. Three coins were prepared for each sampling group (ham, bologna, control and treatment) and samples for all 5 subsequent time point analyses were prepared simultaneously. The samples were then inoculated by dipping in the inoculum bath, using a 21 cm diameter stainless steel mesh basket for 30 sec and then were allowed to dry on sterile filter paper in a laminar airflow hood for 15 min prior to packaging.

Three coins from each sample group were used as time zero samples, held at 4 °C and sampled within the next 8 h. The remaining sample units were divided into groups of 3 for each time point and vacuum packed in heat-sealed low O_2 permeable PVDC plastic bags (<15 $cm^3/cm^2/day.atm.23$ °C) using a Bizerba model GM 2002 vacuum packaging machine (Mississauga, ON). The samples were then placed at 8 °C to incubate for later sampling. Sampling was conducted weekly for 4 weeks after inoculation or until the test organism had reached levels of $\geq 10^7$ $CFU.cm^{-2}$.

At each time point the coins from each sampling group were removed from their packages and placed separately in a stomacher bag with 90 mL of 0.1% peptone and massaged (Stomacher 400, A.J. Seward, Canlab, Toronto, ON) for 2 min, to produce a 10^{-1} dilution. The mixed samples were then serially diluted, 100 µL in 9.9 mL 0.1% peptone, to produce dilutions of 10^{-3} and 10^{-5}. The 3 dilutions prepared were then plated, in duplicate, on the appropriate selective medium and SMA using the spiral plater. The selective media were incubated under appropriate conditions (see above) for the individual test organisms to

allow for specific enumeration, and the SMA plates were incubated anaerobically to yield total anaerobic numbers (TAN). The number of colony forming units (CFU) per gram of sample were converted to CFU.cm^{-2} of sample surface.

5.3.4. Data Treatment

The aim of the analysis of the data generated by this experiment was to determine if the presence of lysozyme, nisin and EDTA in the treated samples resulted in significantly different bacterial populations of the test organism and TAN compared to inoculated control samples without the inhibitors.

The data were first screened to determine if there was a practical difference between the bacterial population of the control and treated samples at each time point during storage. It was deemed that a practical difference in population levels was present if the difference between the means for the control and treatment was equal to or greater than one log CFU.cm^{-2}. A value of one log was chosen for practical significance as differences of one order of magnitude are generally regarded as being of microbial significance (Jarvis, 1989; Gill and Baker, 1998). If the difference between the two means were of practical significance, a two way t-test with $\alpha = 0.05$, was conducted to determine whether the difference between the means was statistically significant. For results to have been considered significant, the difference between means of control and treatment met the conditions of both practical and statistical significance.

5.4. Results

The results of the experiments using *B. thermosphacta* and *Lc. mesenteroides* were interpreted with the knowledge that these were initially packaged under incomplete vacuum Once this was discovered, the samples were immediately repackaged appropriately under vacuum.

On both control (inoculated but no inhibitor) and treated (with inhibitor) samples, populations of *Lb. sakei*, *Lc. mesenteroides* on ham, *L. monocytogenes* on ham, and *Ser. grimesii* on both meats, rose to exceed 7 log CFU.cm^{-2} within the first week of storage at 8 °C. No significant difference was observed between bacterial numbers recovered from control and treated samples of ham and bologna inoculated with *Lb. sakei*, *Ser. grimesii* and *Sh. putrefaciens.*

On both ham and bologna samples treated with lysozyme, nisin and EDTA, no *B. thermosphacta* were recovered (< 1.81 log CFU.cm^{-2}), up to and including 4 weeks of storage. In contrast, on control samples *B. thermosphacta* populations rapidly increased (Figs. 5.1 and. 5.2), reaching numbers > 7 log CFU.cm^{-2} within one week of storage at 8 °C.

On ham the numbers of *E. coli* O157:H7 remained constant at approximately 4 log CFU.cm^{-2} on treated samples, whereas the numbers on control samples increased by 3 log CFU.cm^{-2} (Fig. 5.3). The *E. coli* O157:H7 population on treated bologna dropped by approximately 1 log CFU.cm^{-2} over the first 3 weeks of storage before suddenly rising to 8.33 log CFU.cm^{-2} by the fourth week. On control bologna the population of *E. coli* O157:H7

rose steadily to 7.03 log CFU.cm^{-2} (Fig. 5.4).

Although initial numbers of *Lb. curvatus* on control and treated meats were not significantly different, subsequent numbers of viable organisms on both ham and bologna were significantly lower on the treated samples, up to and including 3 weeks of incubation. The difference in *Lb. curvatus* population level between control and treated samples on ham was 1 log CFU.cm^{-2} at weeks one and two, which increased to 2 log CFU.cm^{-2} at week 3 (Fig. 5.5). On bologna the difference in population levels was approximately 2 log CFU.cm^{-2} at weeks one, 2 and 3 (Fig. 5.6).

The numbers of *Lc. mesenteroides* recovered from ham was observed to be lower on treated samples (Log CFU.cm^{-2}, 2.22 sd 0.39) than control samples (Log CFU.cm^{-2}, 4.75 sd 0.01) at time zero. However, on ham no difference in bacterial populations was observed between control and treated samples from week one onward. The *Lc. mesenteroides* populations on treated bologna were significantly lower by at least 1 log CFU.cm^{-2} up to week 3 of storage (Fig. 5.7).

No significant difference was observed between *L. monocytogenes* populations on control or treated samples of ham. On bologna, the population of *L. monocytogenes* was at least 1 log CFU.cm^{-2} lower on treated samples up to two weeks of incubation (Fig. 5.8).

The results also were taken to indicate that the growth of *S. typhimurium* on treated bologna or ham was not significantly different from controls. The numbers of *S. typhimurium*

on both control and treated ham and bologna dropped by 1.5 to 2 log CFU.cm^{-2} between the first week and week 4 (Figs. 5.9 and 5.10).

5.5. Discussion

The results generated from this experiment indicate that the addition of 500 mg.kg^{-1} 1:3:: lysozyme:nisin and 500 mg.kg^{-1} EDTA to ham and bologna mix prior to cooking may restrict the growth of *B. thermosphacta*, *E. coli* O157:H7 and *Lb. curvatus*. Addition of the same antimicrobial agents to bologna also restricted the growth of *Lc. mesenteroides* and *L. monocytogenes*. The presence of the antimicrobials had no apparent effect upon the growth of *Lb. sakei*, *Lc. mesenteroides*, *L. monocytogenes* on ham, or *S. typhimurium*, *Ser. grimesii*, and *Sh. putrefaciens* on either meat. Results obtained with *Lc. mesenteroides* and *L. monocytogenes* inoculated ham as well as those with *Lb. sakei*, *Ser. grimesii*, and *Sh. putrefaciens* are not presented here. Vacuum packed cooked ham and bologna did not appear to be a favorable environment for *S. typhimurium* as the numbers of this organism decreased on controls over 4 weeks of storage at 8 °C.

It was observed that the numbers of *B. thermosphacta* and *Lc. mesenteroides* recovered from both ham and bologna that was treated with inhibitors were lower than those recovered from controls at T= 0. Since both control and inhibitor treated samples were inoculated from the same source this would indicate that the antimicrobials had a bactericidal effect upon these organisms. The treatment reduced *B. thermosphacta* numbers below detectable levels (<1.81 Log CFU.cm^{-2}) and inhibited the growth of any survivors for four weeks of incubation. The treatment also reduced the initial population of *Lc. mesenteroides*

on both ham and bologna. Though *Lc. mesenteroides* populations recovered within a week on ham, the growth rate on bologna was reduced and it took 2 weeks for populations on inhibitor-treated samples to reach the same levels as controls. The lack of vacuum conditions for the first two days of incubation supports the conclusion that the inhibitor was highly effective against *B. thermosphacta*, as the growth of this organism at cured meat pH is unrestricted under poor vacuum (Borch, Kant-Muermans & Blixt, 1996). However, the poor vacuum conditions may have resulted in an increased growth rate for *B. thermosphacta* on controls and altered the composition of the background flora. For the facultative anaerobe *Lc. mesenteroides*, the lack of a vacuum may have reduced inhibitor effects at week one and altered the composition of the background flora, but this would not have affected the initial population levels.

The lysozyme-nisin treatment appeared to reduce the growth rate of *Lb. curvatus* on both ham and bologna but did not appear to inhibit growth by other LAB. Similar results were obtained with *L. monocytogenes*, though growth was only significantly inhibited on bologna.

The inhibitor treatment reduced the growth of *E. coli* O157:H7 on ham. On bologna, treatment was a little more effective but resulted in only a drop of 1 log CFU.cm^{-2} in *E. coli* O157:H7 numbers over the first 3 weeks of storage. This indicates that on bologna the treatment had a lethal effect on *E. coli* O157:H7 cells under vacuum storage. The cause of the sudden rise in the *E. coli* O157:H7 numbers on treated bologna between week 3 and week 4 is unknown. If due to a failed seal on the packaging this could indicate that the treatment was ineffective under aerobic conditions. It would not seem likely that the rise was a result

of reduced antimicrobial activity of lysozyme-nisin over time as the same result was not observed on ham.

It was observed in all experiments, except those with *B. thermosphacta* and *E. coli* O157:H7, that the total anaerobic numbers (TAN), from incubation on SMA, closely paralleled the population levels on the selective media. This indicates that, even when inhibited by lysozyme-nisin treatment these organisms still formed the dominant microflora on the product. With *B. thermosphacta* it was observed that TAN from both control and treated samples rapidly rose to spoilage levels on both ham and bologna. This suggests that in the absence of *B. thermosphacta*, other organisms (probably LAB) were able to replace it as the dominant flora. On ham, *S. typhimurium* remained the dominant flora on both control and treated samples, until week 4 when TAN were observed to be significantly greater on controls. On treated bologna *S. typhimurium* formed the dominant flora over the period of storage. However, on inoculated control bologna the population of *S. typhimurium* was consistently less than the TAN.

In a number of the experiments described here it was noted that there was a difference between the activity of the inhibitory treatment against a particular organism on ham as opposed to bologna. The greater effectiveness on bologna may have resulted from the presence or absence of additional restrictive conditions, such as greater residual nitrite levels (stability)or lower water activity (Holley, Doyon, Fortin, Rodrigue & Carbonneau, 1996). Our analysis rules out any effect of pH. The pH values of cooked ham and bologna measured in our study (6.52 and 6.49, respectively) were typical of commercial products. Other authors

(Cutter and Siragusa, 1997; Murray and Richard, 1997) have observed that nisin activity may be rapidly reduced after addition to meat products. It was speculated that the difference between the effectiveness of the treatment between ham and bologna may result from a difference in the ability of the antimicrobial agents to persist in the product.

The results of these experiments indicate that a lysozyme, nisin and EDTA combination treatment may be of use in controlling the colonization and growth of select organisms of concern in safety and spoilage of cured meat products. Homofermentative LAB and *Leuconostoc* spp. (Holley, 1997b; Yang and Ray 1994) are most frequently responsible for spoilage of these types of products. Since these experiments were conducted at 8 °C to allow rapid evaluation of inhibitory effects, it can be expected that the observed inhibition of microbial growth would be enhanced by incubation at lower temperatures, such as 4 °C, which is commonly recommended for storage of cured meat products.

Future research is required to examine the effect of this treatment on a wider range of organisms. Additionally, investigation of alternative treatment delivery systems may be warranted.

The cooking of the product may reduce the activity of treatment agents incorporated into the product. At pH 2 nisin can withstand prolonged heating at 115 °C, though stability decreases as pH increases and some food components can be protective. Pasteurization of milk for cheese production does not reduce nisin activity by more than 20% (Delves-Broughton et al., 1996). The enzyme activity of lysozyme can be reduced by pasteurization

temperatures, though the extent of inactivation is dependent upon the environment (Proctor and Cunningham, 1988). In contrast, Ibrahim (1998) has shown that the antimicrobial activity of lysozyme may be enhanced by heating though enzymatic activity is reduced.

If heating does reduce antimicrobial activity of the antimicrobial agents then one approach may be to incorporate the treatment agents into a packaging system to be applied to the surface of the product after thermal treatment. This is an attractive alternative since it has been observed that the growth of microbial populations on cured meats occurs on the surface of the products and colonization results from post-heat treatment contamination (Holley, 1997a,b). Sequestering of the treatment agents in a packaging material or film would localize the agent concentration at the site of microbial activity, which would probably result in more efficient activity against sensitive organisms.

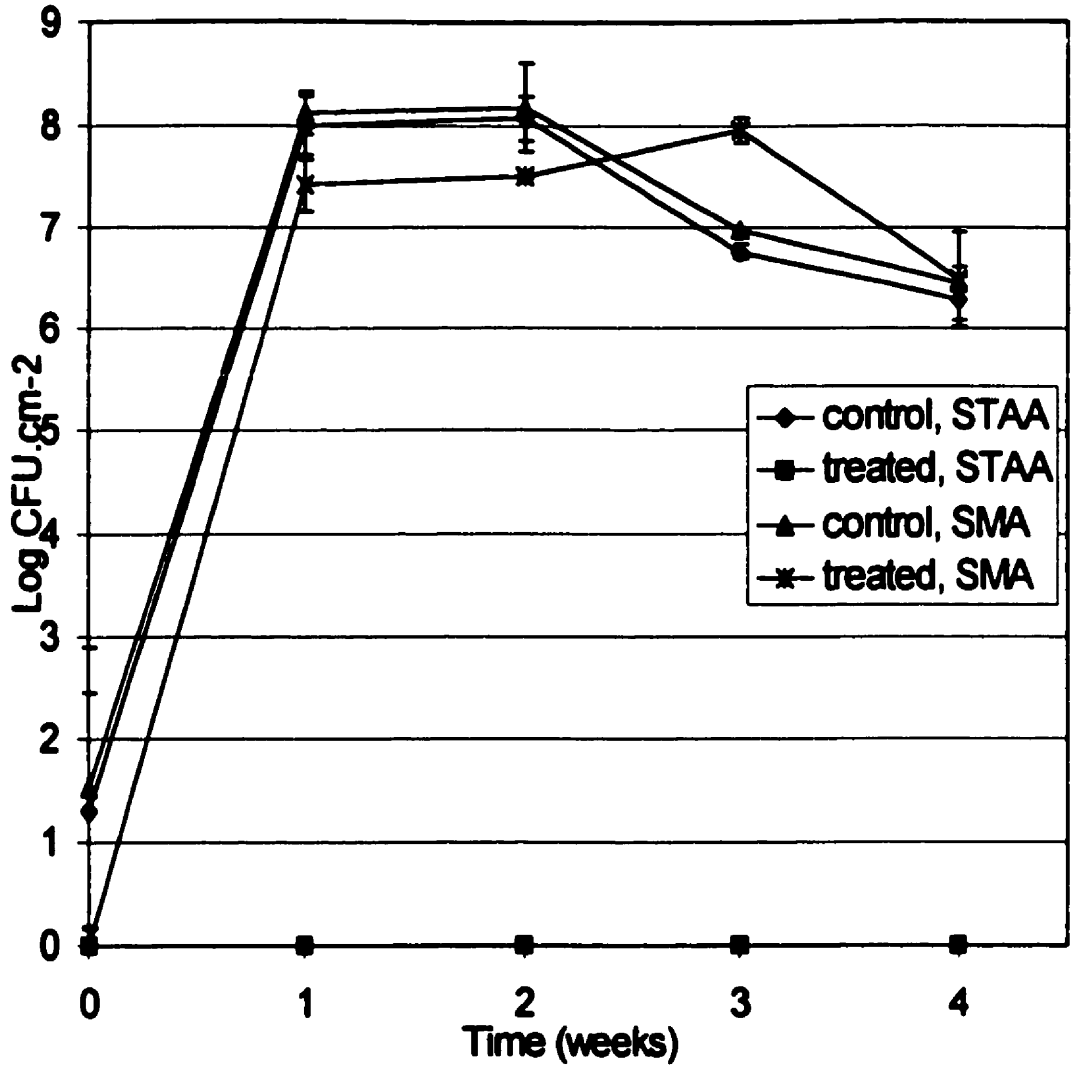

Figure 5.1. Growth of *Brochothrix thermosphacta* on ham formulated with lysozyme:nisin::1:3 (500 mg.kg^{-1}) plus EDTA (500 mg.kg^{-1}) (treated), or without inhibitor (control). All samples were fully cooked before inoculation with the test organism, vacuum packaged and stored at 8 °C. Organisms were recovered on standard methods agar (SMA) or streptomycin thallus acetate actidione agar (STAA). Vertical bars represent one standard deviation interval. Data points without visible vertical bars have a standard deviation of <0.1 CFU.cm^{-2}.

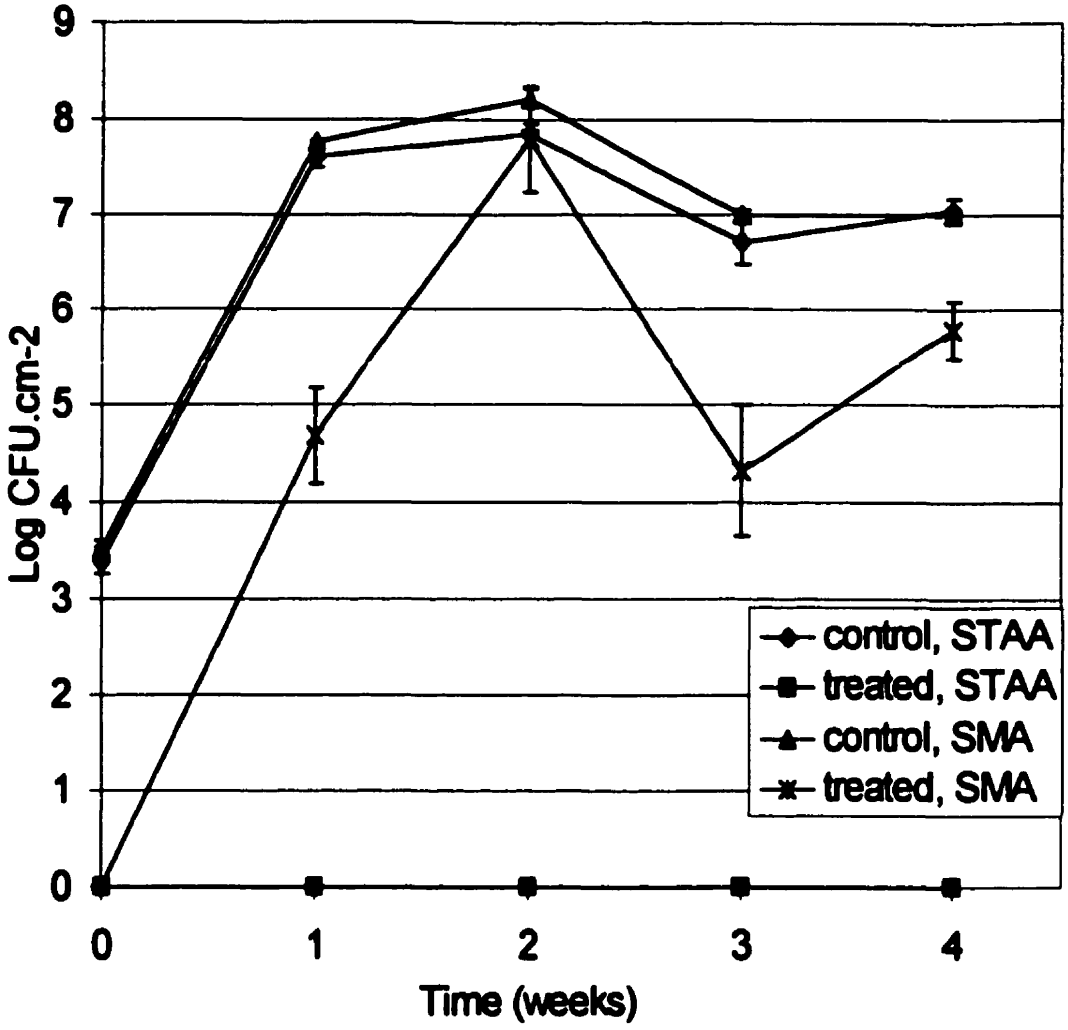

Figure 5.2. Growth of *Brochothrix thermosphacta* on bologna formulated, cooked, inoculated, packaged, and stored as described in Fig. 5.1.

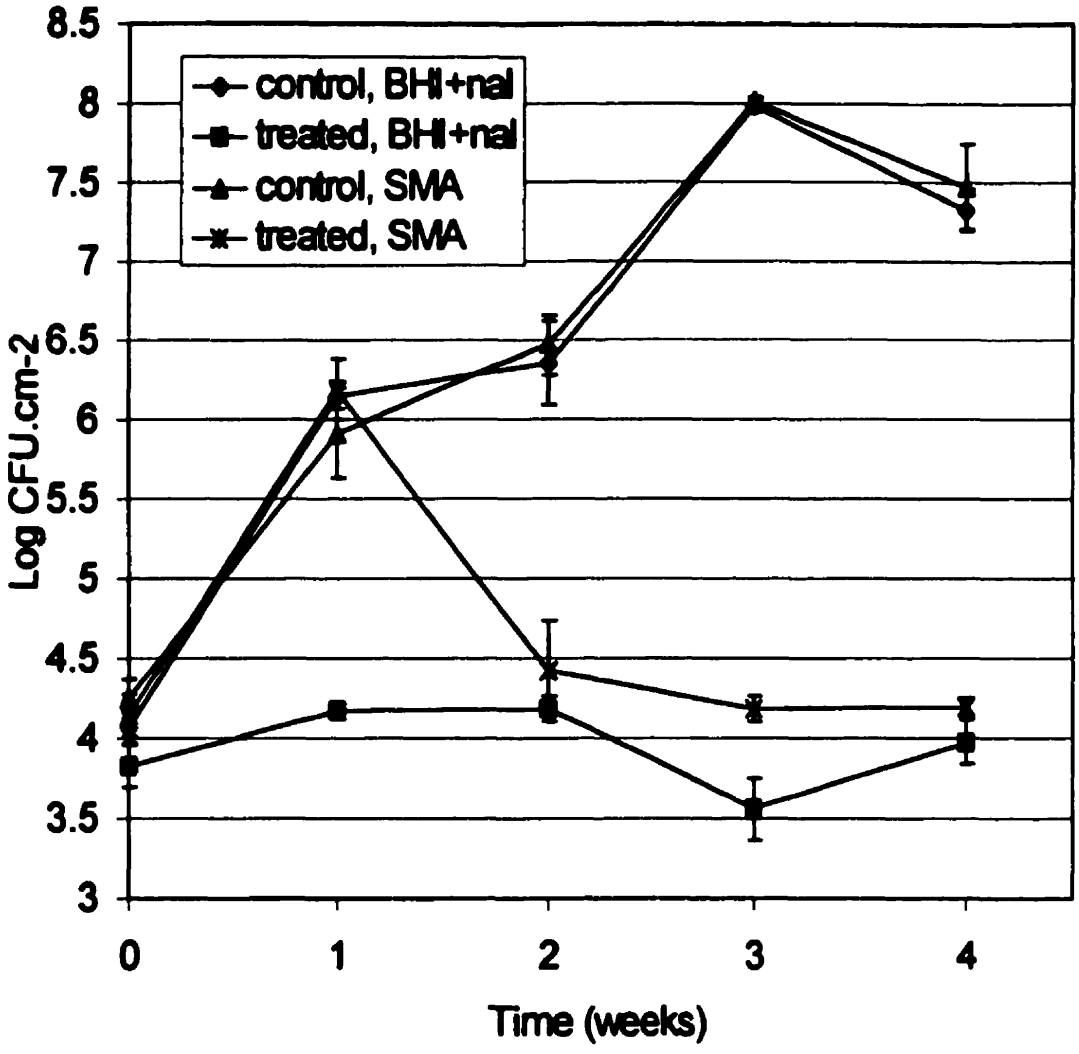

Figure 5.3. Growth of *Escherichia coli* O157:H7 on ham formulated with (treated), or without inhibitor (control) as in Fig. 5.1. All samples were fully cooked before inoculation with *Escherichia coli* O157:H7, vacuum packaged and stored at 8 °C. Organisms were recovered on standard methods agar (SMA) or brain heart infusion agar containing 30 ppm nalidixic acid (BHI+nal).

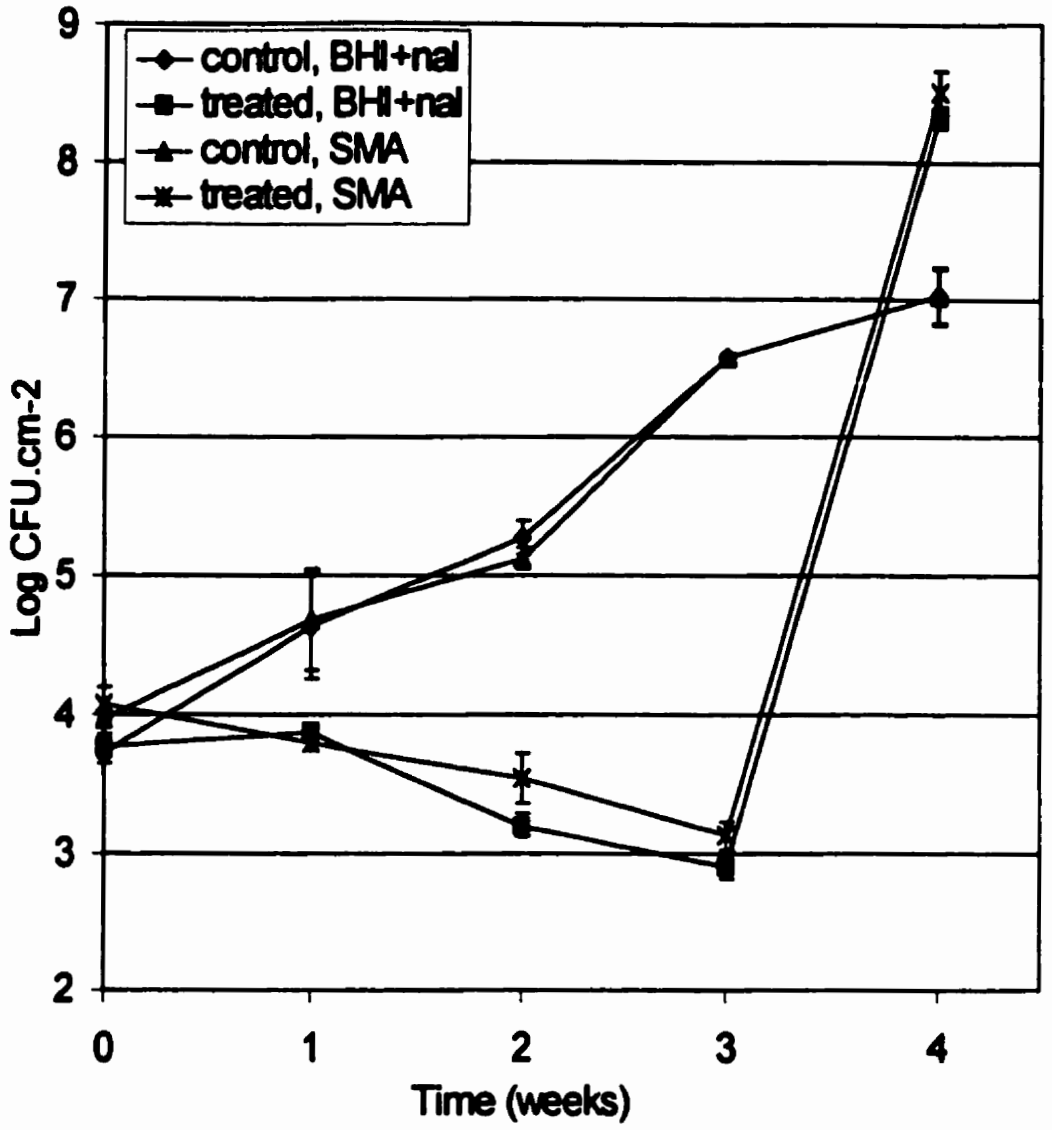

Figure 5.4. Growth of *Escherichia coli* O157:H7 on bologna formulated, cooked, inoculated, packaged, and stored as described in Fig. 5.3.

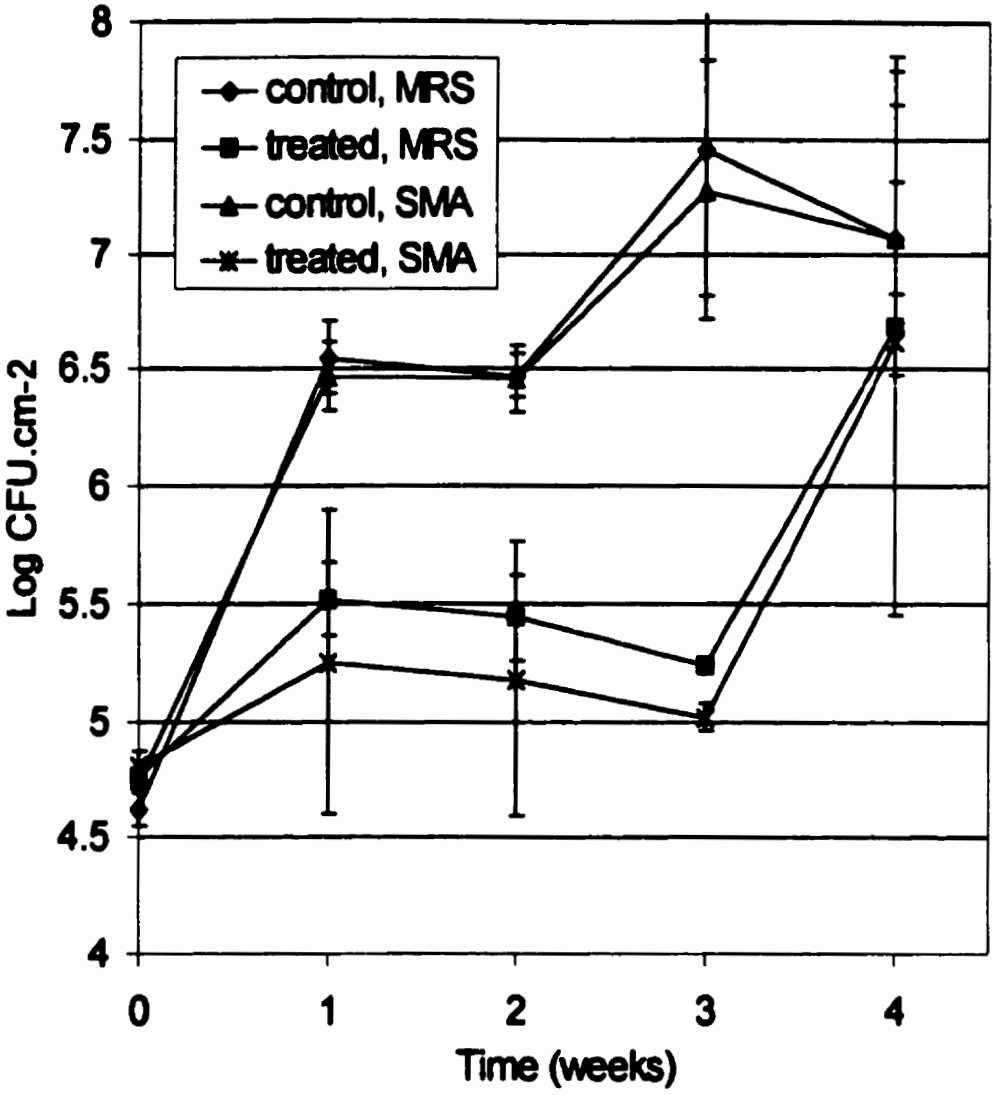

Figure 5.5. Growth of *Lactobacillus curvatus* on ham formulated with (treated), or without inhibitor (control) as in Fig. 5.1. All samples were fully cooked before inoculation with *Lactobacillus curvatus*, vacuum packaged and stored at 8 °C. Organisms were recovered on standard methods agar (SMA) or deMan, Rogosa and Sharpe agar (MRS).

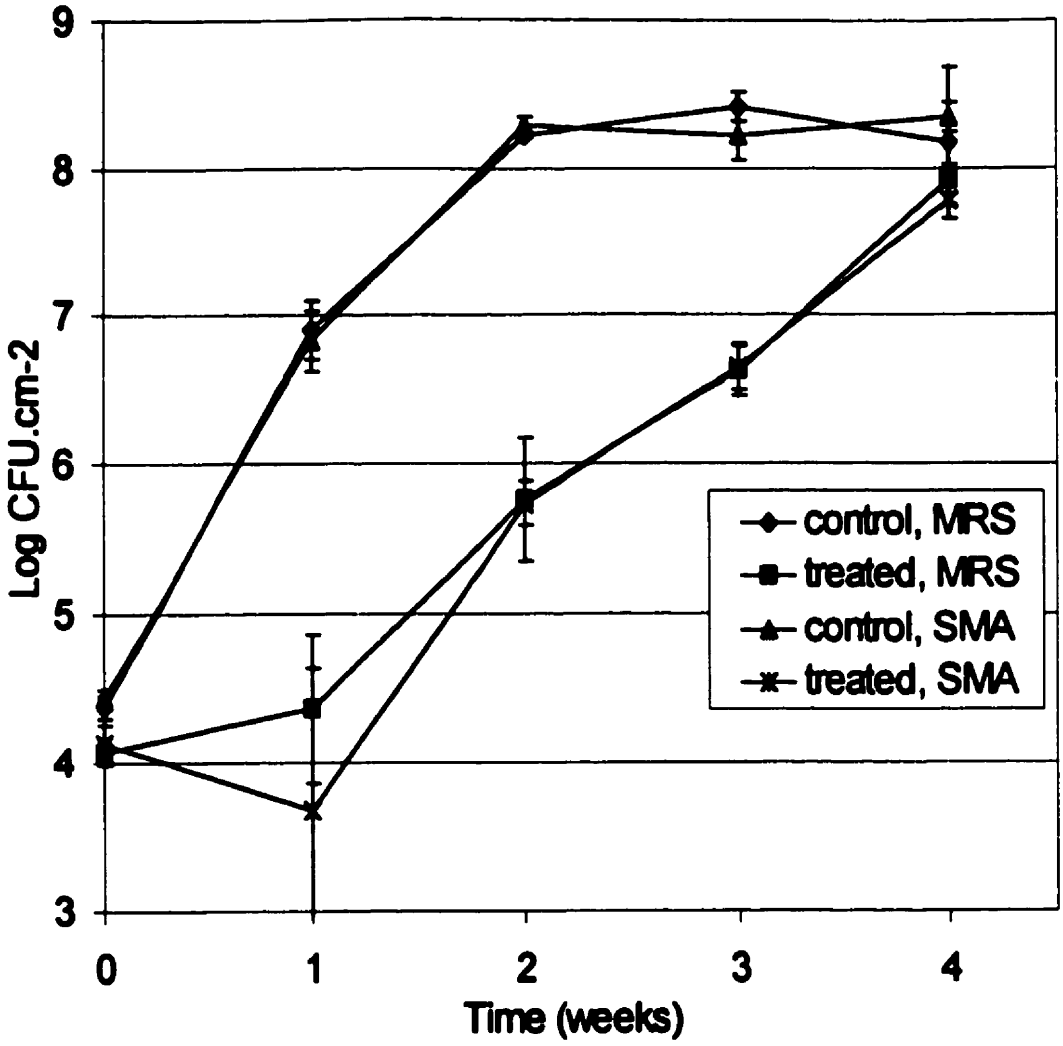

Figure 5.6. Growth of *Lactobacillus curvatus* on bologna formulated, cooked, inoculated, packaged and stored as described in Fig. 5.5.

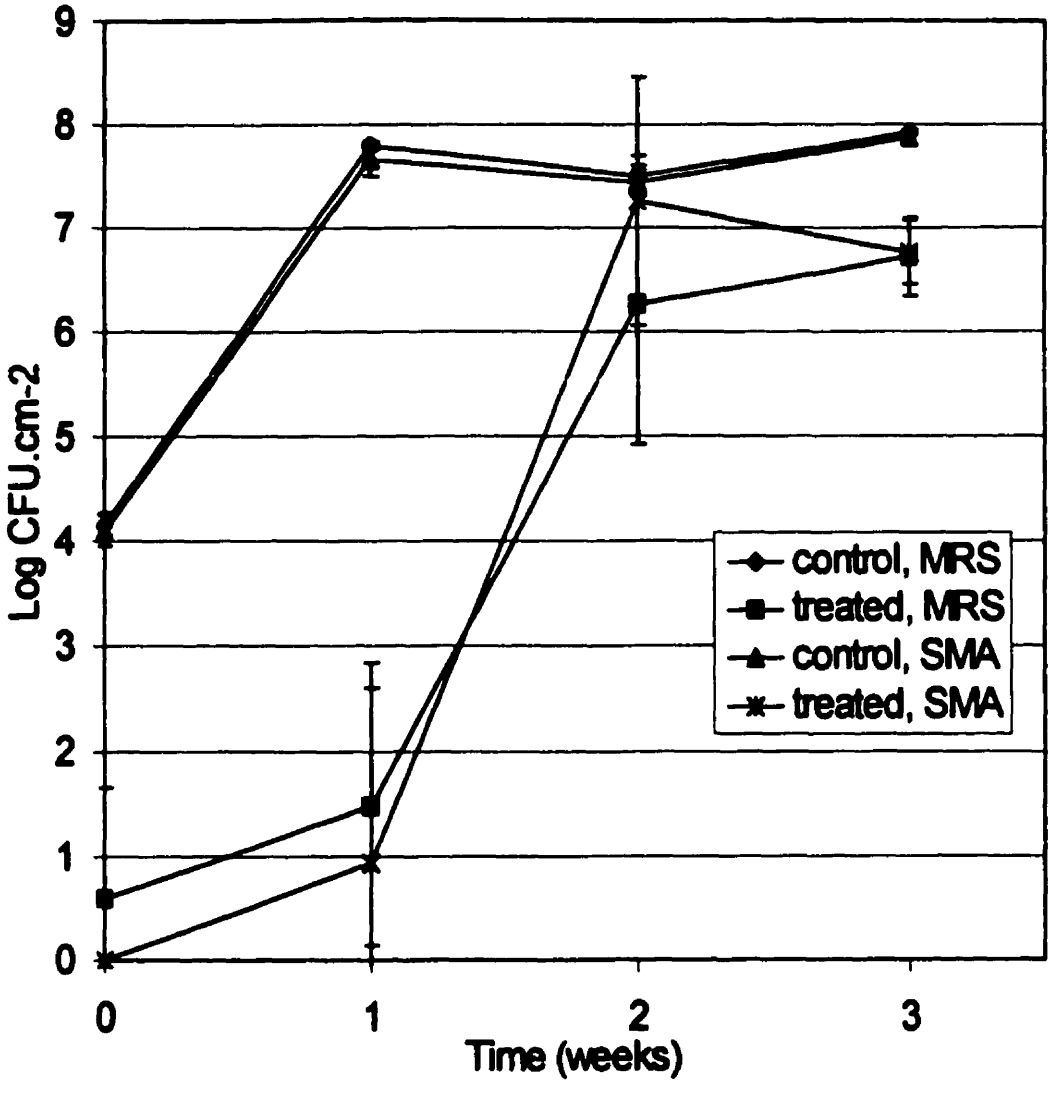

Figure 5.7. Growth of *Leuconostoc mesenteroides* on bologna formulated with (treated), or without inhibitor (control) as in Fig. 5.1. All samples were fully cooked before inoculation with *Leuconostoc mesenteroides*, vacuum packaged and stored at 8 °C. Organisms were recovered on standard methods agar (SMA) or deMan, Rogosa and Sharpe agar (MRS).

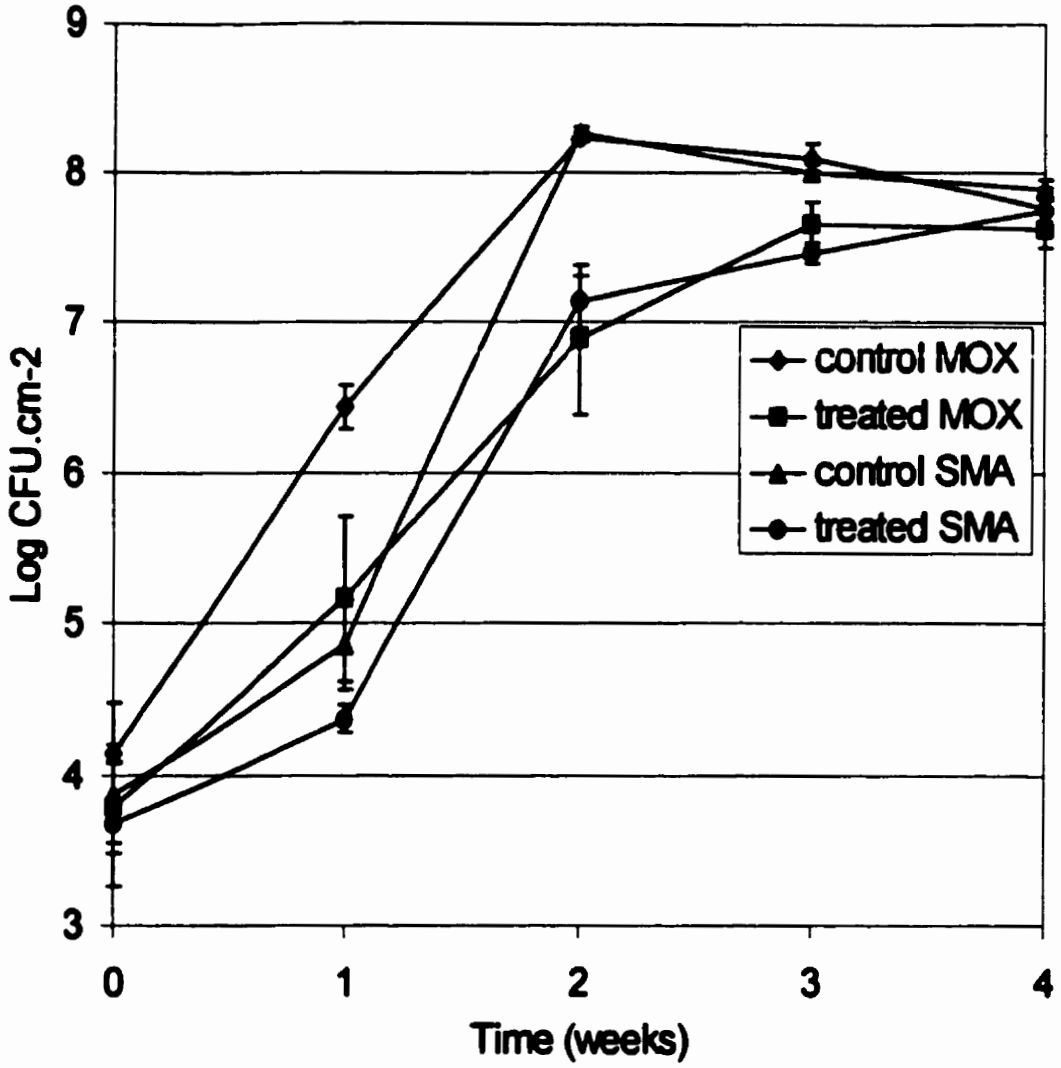

Figure 5.8. Growth of *Listeria monocytogenes* on bologna formulated with (treated), or without inhibitor (control) as in Fig. 5.1. All samples were fully cooked before inoculation with *Listeria monocytogenes*, vacuum packaged and stored at 8 °C. Organisms were recovered on standard methods agar (SMA) or modified oxford agar (MOX).

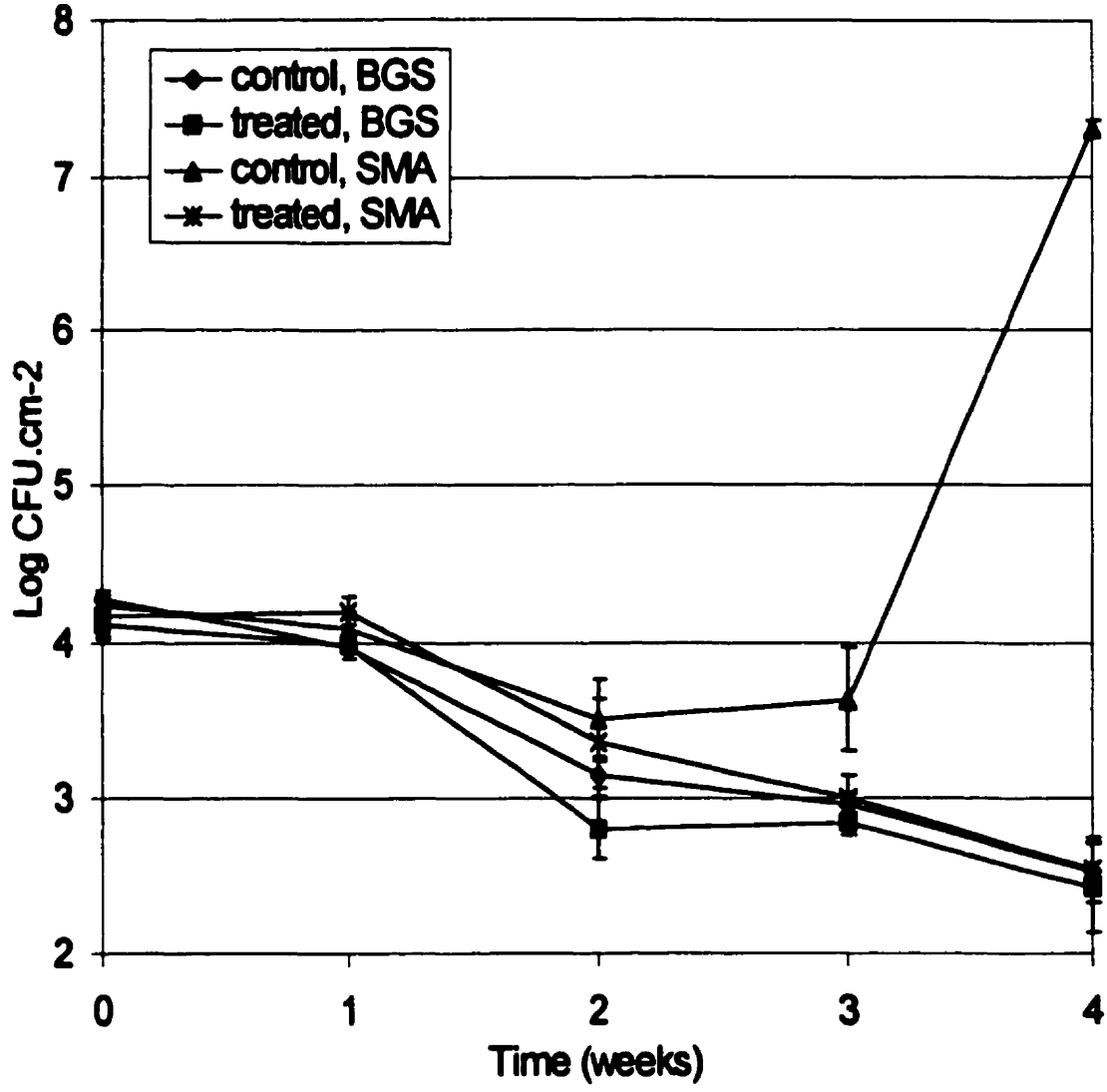

Figure 5.9. Growth of *Salmonella typhimurium* on ham formulated with (treated), or without inhibitor (control) as in Fig. 5.1. All samples were fully cooked before inoculation with *Salmonella typhimurium*, vacuum packaged and stored at 8 °C. Organisms were recovered on standard methods agar (SMA) or brilliant green agar with sulfadiazine (BGS).

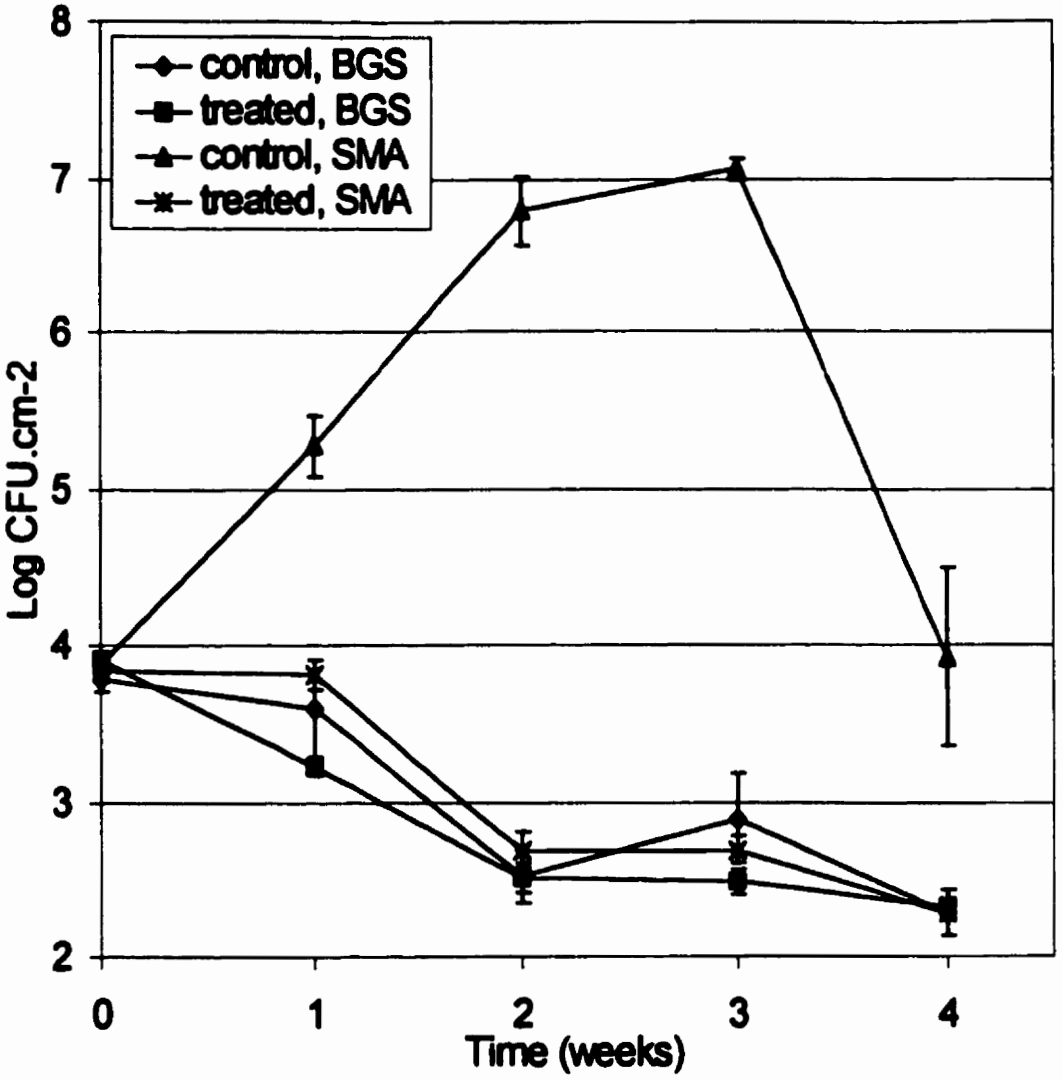

Figure 5.10. Growth of *Salmonella typhimurium* on bologna formulated, cooked, inoculated, packaged, and stored as described in Fig. 5.9.

6. Surface application of lysozyme, nisin, and EDTA to inhibit spoilage and pathogenic bacteria on ham and bologna

6.1. Abstract

Cooked ham and bologna sausage received one of three treatments: no coating (control); coating with a 7% (w/v) gelatin gel (gel-control), or coating with a 7% gelatin gel containing 25.5 g/liter lysozyme:nisin::1:3 plus 25.5 g/litre EDTA (gel-treated). The samples were then inoculated with one of six test organisms: *Brochothrix thermosphacta; Escherichia coli* O157:H7; *Lactobacillus sakei; Leuconostoc mesenteroides; Listeria monocytogenes,* or *Salmonella typhimurium*. Inoculated samples were vacuum packed and stored at 8 °C for 4 weeks.

The antimicrobial gel treatment had a bactericidal effect up to 4 log CFU/cm^2 on the Gram positive organisms tested and inhibited the growth of these organisms over the 4 weeks of storage.

The antimicrobial gel treatment also had a bactericidal effect on the growth of *S. typhimurium* over the period of storage.

E. coli O157:H7 growth on ham was reduced by 2 log CFU/cm^2 following treatment with both antimicrobial-containing and non-antimicrobial gels over the 4 week storage period. No effect was observed upon the growth of *E. coli* O157:H7 on bologna.

6.2. Introduction

Previous studies (Chapter 5) demonstrated that the addition of 500 mg/kg lysozyme:nisin::1:3 plus 500 mg/kg ethylenediamine tetraacetate (EDTA) to ham or bologna sausage batter prior to cooking, reduced the number of viable cells of *Brochothrix (B.) thermosphacta*, *Escherichia (E.) coli* O157:H7, and *Lactobacillus (Lb.) curvatus* on vacuum packed sausage stored at 8 °C. The addition of these antimicrobials to bologna batter also resulted in reduced numbers of *Leuconostoc (Lc.) mesenteroides* and *Listeria (L.) monocytogenes*. The treatment was found to be ineffective against *Lactobacillus (Lb.) sakei*, *Salmonella (S.) typhimurium*, *Serratia (Ser.) grimesii*, or *Shewanella (Sh.) putrefaciens*. Davies et al. (1999) reported that the addition of 25 mg/kg pure nisin (equivalent to 1000 mg/kg of a 2.5 % commercial preparation) to bologna batter increased the time required for spoilage by a mixed lactic acid bacterium (LAB) population from 7 days to > 50 days when vacuum packed at 8 °C.

A number of authors have published studies of nisin and nisin/chelator based antimicrobial treatments directed toward organisms attached to the surface of meat. Many of these studies have indicated that organisms are more resistant to the antimicrobial treatments when in a food system compared to when cells are in liquid suspension. Mahadeo and Tatini (1994) observed that the numbers of *L. monocytogenes* subjected to 100 IU/ml of nisin (prepared from a 2.5% commercial preparation of nisin, with activity of 10^6 IU/g) in suspension were reduced by >4 log CFU/ml, while the same treatment applied to cells attached to squares of turkey skin reduced *L. monocytogenes* by approximately 1 log CFU/ml. Treatment of beef cubes with 50 µg/mL of pure nisin and 50 mM EDTA reduced attached

S. typhimurium or *E. coli* O157:H7 by less than 0.5 log CFU/cm^2 (Cutter and Siragusa, 1995). When beef was treated with solutions of 2% (w/v) lactic acid or 2% (w/v) polylactic acid alone, or in combination with 200 IU/ml of pure nisin, a bactericidal effect was observed against the *Enterobacteriaceae* and *Pseudomonas* spp. Addition of nisin did not increase the effectiveness of the acid treatments, and none of the treatments affected the numbers of LAB recovered (Ariyapitipun et al., 1999). Shefet et al. (1995) demonstrated that immersion of turkey skin for 30 min in a solution of 100 μg/ml nisin, 5 mM EDTA, 3 % (w/v) citric acid and 0.5 % Tween 20, resulted in a 4.9 log CFU/ml greater reduction of attached *S. typhimurium* cells compared to 20 ppm chlorine. Application of the same treatment to whole turkey drumsticks increased the shelf life by 1.5 to 3 days at 4 °C (Shefet et al., 1995). Treatment of vacuum packed beef at 4 °C with 5000 IU/ml nisin or 20 mM EDTA alone or combined, had no effect on *E. coli* O157:H7 inoculated at 3 log CFU/cm^2 (Zhang and Mustapha, 1999). *L. monocytogenes* on beef subjected to the same treatment was reduced 2 log CFU/cm^2 by nisin and 1 log CFU/cm^2 by nisin and EDTA. Since the numbers of *L. monocytogenes* recovered from controls and treated beef was constant over the storage period of 30 days, no conclusions can be drawn about the effects on a population of cells capable of recovery (Zhang and Mustapha, 1999).

The effectiveness of nisin treatment of meat was increased by its immobilization in gel. Cutter and Siragusa (1997) showed that 0.1 mg/ml pure nisin incorporated into a calcium alginate gel was more effective against *B. thermosphacta* growing on ground beef than unbound nisin. Fang and Lin (1995) demonstrated greater reduction of *L. monocytogenes* in cooked pork tenderloins when nisin was immobilized in a calcium alginate gel. These

studies also demonstrated that the activity of nisin in the meat persisted longer when immobilized in alginate gel. Nisin added to ground pork at 300, 1500 and 3000 IU/ml has been shown to have a bactericidal effect on *Listeria innocua*; however, in all cases the population of surviving cells increased at a rate similar to controls after 2 days storage at 4 °C, coincident with the end of lag phase in controls (Murray and Richard, 1997).

Holley (1997a,b) demonstrated that the growth of microbial populations on vacuum packed cured meats occurs on the surface of the products and colonization results from post-heat treatment contamination. It was hypothesized that the effectiveness of a lysozyme, nisin and EDTA treatment could be enhanced by applying the antimicrobial treatment to the surface of the cooked meats. This could be achieved by dipping products in an antimicrobial bath, or by application of an edible gel, or by use of a packaging system that would sequester the antimicrobial agents at the site of microbial activity

In the present experiments, a 7% (w/v) gelatin gel was used to immobilize the antimicrobials. The gelatin gel provided a 1 mm thick, transparent and colourless coating for ham and bologna, with little difference in the visual appearance between gel-coated and control samples.

Preliminary trials against *B. thermosphacta*, *Lb. sakei* and *L. monocytogenes* were conducted during which lower concentrations of lysozyme, nisin and EDTA were delivered by dipping meat in an aqueous suspension or by coating in a 7% gelatin gel (results not shown). Treatment concentrations of 500 mg/liter lysozyme:nisin::1:3 plus 500 mg/liter

EDTA and 2000 mg/liter lysozyme:nisin::1:3 plus 1000 mg/liter EDTA were found to be less effective than when 500 mg/kg lysozyme:nisin::1:3 plus 500 mg/kg EDTA were added directly to the meat batter (Chapter 5). Delivery of antimicrobials by dipping in an aqueous suspension was observed to be less effective at the same concentration than by delivery in a 7% gelatin gel; thus, dipping in an aqueous solution was rejected as a delivery method for further trials. The trials described in this report used 25.5 g/liter lysozyme:nisin::1:3 plus 25.5 g/liter EDTA incorporated into a surface applied 7% gelatin gel. This is equivalent in concentration to 450 mg/kg of antimicrobial added directly and mixed with the sausage batter (the calculation is based upon 10 g of sausage being coated with 0.2 g of gelatin gel).

6.3. Materials and Methods

6.3.1. Cultures used, incubation conditions and selective media

B. thermosphacta B2 was from Dr. G. Greer, Agriculture and Agrifood Canada (AAFC) Research Station, Lacombe, AB (brain heart infusion broth, BHI, aerobic). *E. coli* O157:H7 strain E318 was a human clinical isolate provided by Dr. R Johnson, Health Canada, Guelph, ON (BHI, aerobic). *Lb. sakei* #7 and *Lc. mesenteroides* #11 were isolated from spoiled cured meats (Holley et al., 1996) (all purpose tween broth, APT, anaerobic). *L. monocytogenes* was from the Univ. Manitoba, Department of Food Science culture collection (BHI, aerobic). *S. typhimurium* #98 was from the AAFC culture collection, Guelph ON (BHI, anaerobic).

Cultures were transferred monthly on agar slants at 4 °C and maintained as frozen glycerol stocks. For experimental use cultures were streak-plated once a week and cultures

for experiments were inoculated into media from a single colony and incubated overnight at 24 °C under the appropriate atmospheric conditions. All cultures were maintained in either APT or BHI media, except for *Lb. sakei* #7 which was maintained on M17 agar, but grown in APT broth. The MI7 medium was used for the maintenance of *Lb. sakei* since the glycerol phosphate it contains prevented rapid reduction in pH.

Organisms were recovered from inoculated meat samples using the following selective media: *B. thermosphacta*, aerobic, streptomycin thallous acetate actidione agar (STAA); E. coli, MaConkey sorbitol agar with 0.050 mg/L cefixime and 2.5 mg/L tellurite (ctSMAC). *Lb. sakei* and *Lc. mesenteroides* , anaerobic, deMan, Rogosa and Sharpe agar (MRS); *L. monocytogenes*, aerobic, modified oxford medium agar (MOX); *S. typhimurium*, anaerobic, brilliant green agar with sulfadiazine (BGS). The inoculated ctSMAC medium was incubated for 24 h at 37 °C prior to enumeration; all other media were incubated for 48 h at 24 °C.

6.3.2. Materials

Commercially prepared chopped ham mix (12.5% meat protein, pork, water, salt, sugar, dextrose, sodium phosphate, carrageenan, sodium erythorbate and sodium nitrite) and bologna emulsion (pork, mechanically separated turkey, chicken or pork, beef or beef byproducts, water, wheat flour, potato starch, salt, dextrose, spices, sodium erythorbate, sodium nitrite, and smoke) were kindly provided by Maple Leaf Meats, Winnipeg, MB. Sausage casings (hog intestine, 30 mm diameter) were from Canada Compound Western, Winnipeg, MB. Sausage casings were stuffed with meat batters using a 9 liter capacity mechanical sausage stuffer from F. Dick Gmbh, Germany. Knox Gelatin distributed by

Tomas J. Lipton Ltd., Toronto, ON was used. Low oxygen permeable polyvinylediene chloride (PVDC) plastic bags were kindly provided by Winpak, Winnipeg, MB (25 x 35 cm, Deli #1). Lysozyme:nisin::1:3 (lot# 9312-26, 23.75 % (w/w) pure lysozyme, 1.69 % (w/w) pure nisin) was premixed and provided by Canadian Inovatech, Abbotsford, BC. The EDTA was from Fisher Scientific, Toronto, ON. The APT broth, BGS and SMA (standard methods agar) were from BBL, Becton-Dickinson, Franklin Lakes, NJ. The BHI broth, granulated agar, MRS broth (Lactobacillus MRS broth), proteose peptone No. 3, oxford medium base, modified oxford antimicrobic supplement (colistin sulfate 10 mg/L, moxalactam 20 mg/L) for MOX, and yeast extract were from Difco, Becton-Dickinson. The M17 agar was supplied by Oxoid Ltd, Basingstoke, U.K. Streptomycin sulphate, cycloheximide (actidione) and thallous acetate were from Sigma-Aldrich, Oakville, ON. An Autoplater 4000 equipped with a CASBA-4 automated counting system was used for surface plating on pre-poured agar media and enumeration (Spiral Biotech, Inc., Bethesda, MD). The anaerobic incubator (model 3640-6) was from the National Appliance Co., Portland, OR, and anaerobic conditions were created by flushing twice with 30% CO_2/ 70 % N_2.

6.3.3. Methods

The ham and bologna sausages used in these experiments were each prepared from a single batch of chopped ham mix or bologna emulsion. The raw meat batters were obtained from the manufacturer on the day of production and stored at -20 °C until the sausages were to be made.

The meat batters were thawed overnight at 4 °C. Using the 9 liter capacity hand-cranked sausage stuffer, sausages of ham and bologna mix were prepared in the natural

casings. The casings were originally salted, but were soaked overnight in deionized water at 4 °C. After stuffing to 12 cm lengths, the sausages were clipped with a metal ring, then cooked to an internal temperature of 69 °C. The heating of the sausages was conducted in a jacketed steam kettle for three intervals of 20 min at 53 °C, 64 °C, and 75 °C. The sausages were then vacuum packed in heat-sealed O_2 barrier (PVDC) plastic bags and stored at -20 °C until required for inoculation.

A culture of the organism to be tested was prepared in 50 mL of BHI or APT broth, as appropriate, and incubated aerobically or anaerobically for 48 h at 24 °C. A dipping bath for inoculation of the control and inhibitor treated samples with each test organism was prepared by diluting 1 ml of culture in 1 liter of 0.1% peptone, resulting in an initial population of approximately 4 log CFU/cm^2 on the meats.

Ham and bologna sausages were thawed overnight at 4 °C and then cut on a sterile board using a flame-sterilized knife into 14 mm "coins". This produced portions of meat 29-31 mm in diam and 14 mm thick, with a surface area (maximum) of 28.73 cm^2, weighing 10 g ± 1 g. Three coins were prepared for each sampling group (ham, bologna, control, gel-control and gel-treated), and samples for all five subsequent time point analyses were prepared simultaneously.

Control samples were inoculated with no further treatment. Gel-control samples were dipped for 5 s in a 7% (w/v) solution of gelatin in sterile water at 45 °C and then cooled for 10 min at 4 °C to set the gelatin. Gel-treated samples were dipped for 5 s in a 7% (w/v)

solution of gelatin in sterile water, to which 25.5 g/liter lysozyme:nisin::1:3 plus 25.5 g/liter EDTA had been added in powder form to yield the required concentration, and then cooled for 10 min at 4 °C to set the gelatin.

The samples were then inoculated with bacteria by dipping in the inoculum bath, using a 21 cm diameter stainless steel mesh basket with 2 mm holes, for 30 sec and then were allowed to dry on sterile filter paper in a laminar airflow hood for 15 min prior to packaging.

Three coins from each sample group were used as time zero samples, held at 4 °C and sampled within the next 8 h. The remaining sample units were divided into groups of three for each time point and vacuum packed in heat-sealed, low O_2 permeable PVDC plastic bags (<15 $cm^3/cm^2/day.atm.23$ °C) using a Bizerba model GM 2002 vacuum packaging machine (Mississauga, ON). The samples were then placed at 8 °C to incubate for later sampling. Sampling was conducted weekly for 4 weeks after inoculation, or until the test organism had reached levels of ≥ 7 log CFU/cm^2.

At each sampling three coins were removed from their packages and placed separately in a stomacher bag with 90 ml of 0.1% peptone and massaged (Stomacher 400, A.J. Seward, Canlab, Toronto, ON) for 2 min, to produce a 10^{-1} dilution. The mixed samples were then serially diluted to produce dilutions of 10^{-3} and 10^{-5}. The three dilutions prepared were then plated in duplicate on the appropriate selective medium and SMA using the spiral plater. The selective media were incubated under appropriate conditions (see above) for the individual

test organisms to allow for specific enumeration. The SMA plates were incubated anaerobically for 48 h. at 24 °C to yield total anaerobic numbers (TAN). The number of CFU/g of sample was converted to CFU/cm^2 of sample surface.

6.3.4. Data Treatment

The data generated from this experiment were analyzed to determine if the presence of lysozyme, nisin and EDTA in the gel-treated samples resulted in significantly different bacterial populations of the test organism and TAN compared to inoculated-control and gel-control samples.

The data were first screened to determine if there was a practical difference between the bacterial population of the control and treated samples at each time point during storage. It was deemed that a practical difference in population levels was present if the difference between the means for the control and treatment was equal to or greater than one log CFU/cm^2. A value of one log was chosen for practical significance as differences of one order of magnitude are generally regarded as being of microbial significance (Gill and Baker, 1998; Jarvis, 1989). If the difference between the two means were of practical significance, a two way t-test with $\alpha = 0.05$ was conducted to determine whether the difference between the means was statistically significant. For results to have been considered significant, the difference between means of control and treatment met the conditions of both practical and statistical significance.

6.4. Results

Sampling of *B. thermosphacta* inoculated ham or bologna treated with gelatin gel containing 25.5 g/liter lysozyme:nisin::1:3 plus 25.5 g/L EDTA (gel-treated) failed to recover any *B. thermosphacta* colonies during storage, whereas the growth of *B. thermosphacta* on controls and samples coated with non-antimicrobial containing gel (gel-control) was unrestricted (Tables 6.1 and 6.2).

On ham or bologna inoculated with *B. thermosphacta*, the number of colonies recovered on SMA from gel-treated samples rose to spoilage levels >7.00 log CFU/cm^2 within two weeks, though no colonies were recovered from this treatment using STAA medium over the period of storage. There was no significant difference between colony numbers on SMA and STAA media from *B. thermosphacta* inoculated control and gel-control samples (Tables 6.1 and 6.2).

Differing treatments had no significant effect on the numbers of *E. coli* recovered initially from ham (Table 6.3) and bologna (Table 6.4).Little change was observed in the numbers of *E. coli* colonies recovered from control samples of ham over the 4 week period of storage (Table 6.3). The numbers of *E. coli* recovered from gel-control and gel-treated samples dropped by approximately 1.5 log CFU/cm^2 over the period of storage, with the reduction beginning in week one of storage for gel-treated samples and week two for gel-control samples. There was no significant difference between the numbers of *E. coli* recovered from samples of bologna at any point during storage (Table 6.4). However, the numbers of *E. coli* recovered on ctSMAC from bologna was reduced by approximately 2 log

CFU/cm^2 compared to the initial inoculum.

The numbers of colonies recovered on SMA from *E. coli* inoculated ham and bologna rose on control and gel-control samples over the 4 weeks of storage (Tables 6.3 and 6.4). The numbers of colonies recovered on SMA from gel-treated samples was observed to drop over the same period.

The numbers of *Lb. sakei* recovered from ham or bologna on gel-treated samples were consistently lower than those on control and gel-control samples (Tables 6.5 and 6.6). *Lb. sakei* on control and gel-control samples rose to >7.5 log CFU/cm^2 within the first week of storage. *Lb. sakei* was not recovered from gel-treated ham until week two of storage and did not exceed 4.5 log CFU/cm^2 during storage (Table 6.5). *Lb. sakei* was only recovered from gel-treated bologna at week three of storage (1.8 log CFU/cm^2), (Table 6.6).

The number of colonies recovered on SMA from *Lb. sakei* inoculated ham and bologna was less than or equal to the number colonies recovered on MRS for control, gel-control and gel-treated samples (Tables 6.5 and 6.6).

The numbers of *Lc. mesenteroides* recovered from ham and bologna control and gel-control samples rose from approximately 4 log CFU/cm^2 to >7.5 log CFU/cm^2 within the first week of storage (Tables 6.7 and 6.8). No *Lc. mesenteroides* colonies were recovered from gel- treated samples except at week three, with 1.30 log CFU/cm^2 on ham (Table 6.7) and 2.39 log CFU/cm^2 on bologna (Table 6.8).

There was no significant difference observed between the numbers of colonies recovered from ham or bologna inoculated with *Lc. mesenteroides* on SMA compared to MRS except at week three for gel-treated samples on ham (Table 6.7).

L. monocytogenes was only recovered from gel-treated ham samples after 3 weeks of storage (Table 6.9). At week three *L. monocytogenes* levels were 2.33 log CFU/cm^2 and at week four were 0.65 log CFU/cm^2, compared to >8.0 log CFU/cm^2 on control and gel-control ham samples (Table 6.9). On gel-treated bologna *L. monocytogenes* was only recovered after 4 weeks of storage at 0.60 log CFU/cm^2 (Table 6.10).

There was no significant difference between the number of colonies recovered on SMA compared with MOX for any of the samples from *L. monocytogenes* inoculated ham or bologna (Tables 6.9 and 6.10).

On ham the numbers of *S. typhimurium* from gel-treated samples were consistently lower than those on control samples from week zero onwards (Table 6.11). Gel-control sample numbers were also significantly lower than control samples at weeks three and four. *S. typhimurium* numbers on control samples remained fairly constant at around 4.0 to 5.5 log CFU/cm^2.

S. typhimurium numbers on control bologna dropped from 4.20 log CFU/cm^2 to 1.49 log CFU/cm^2 over 4 weeks of storage at 8 °C. The numbers of *S. typhimurium* recovered from gel-treated samples was not different from control samples at week zero, but were

significantly lower at weeks one to three, though by week four there was no significant difference on BGS plates due to the decrease in numbers recovered from control samples (Table 6.12).

The only sample for which there was a significant difference between the numbers of SMA and BGS recovered colonies for *S. typhimurium* inoculated ham was gel-control at week three (Table 6.11). On *S. typhimurium* inoculated bologna, the number of colonies recovered on SMA from control and gel-control samples rose over the storage period whereas the number of colonies recovered on BGS declined. However, there was no significant difference in numbers of recovered colonies on SMA compared to BGS from gel-treated samples on bologna (Table 6.12).

6.5. Discussion

In the trials of the antimicrobial gel treatment against the Gram positive organisms, *B. thermosphacta*, *Lb. sakei*, *Lc. mesenteroides*, and *L. monocytogenes*, no colonies were detected on selective or SMA media from samples taken immediately after inoculation, although control and gel-control samples had populations of 3-4 log CFU/cm^2 (Tables 6.1, 6.2 and 6.5 to 6.10). Since the different samples were inoculated from the same source, this would indicate that the antimicrobial-containing gel had a bactericidal effect that reduced the numbers of the Gram positive organisms by at least 4 log CFU/cm^2. The failure of the Gram positive organisms on gel-treated meat to recover to the same levels as on control and gel-control samples, over the 4 week period of storage, indicates that the activity of the antimicrobials was sustained over the storage period.

No significant difference was observed between the initial populations of *E. coli* on ham or bologna and *S. typhimurium* on bologna. The initial numbers of colonies recovered on BGS from *S. typhimurium* inoculated ham were significantly lower for gel-treated samples compared to control samples, indicating a bactericidal effect of approximately 1 log CFU/cm^2 (Table 6.11).

The antimicrobial gel had no apparent effect on *E. coli* inoculated to bologna, as the population on all samples was equally reduced over the period of storage. Coating ham with gel containing, or without antimicrobials appeared to be equally effective in reducing the growth of *E. coli* by 2 log CFU/cm^2 over 4 weeks of storage, while the population detected on ctSMAC-plated controls remained static (Table 6.3). These results indicate that the antimicrobial gel treatment was not effective against *E. coli*. In a previous study in which 500 mg/kg lysozyme:nisin::1:3 plus 500 mg/kg EDTA was added directly to sausage batter (Chapter 5), the antimicrobial treatment was shown to result in static numbers of *E. coli* O157:H7 over the period of storage, whereas the numbers recovered on controls increased. In the previous study, brain heart infusion agar with 30 mg/liter of nalidixic acid was used as the selective media, which may have resulted in the recovery of organisms other than *E. coil* O157:H7 and thus yielded an ambiguous result which was avoided in this study by the use of ctSMAC as the selective medium.

The antimicrobial gel treatment was observed to have a delayed bactericidal effect on *S. typhimurium*. The numbers of *S. typhimurium* recovered were reduced over 4 weeks of storage by 3-4 log CFU/cm^2, while the population slightly increased on control samples and

remained static on gel-control samples. The same conclusion can be reached for *S. typhimurium* growth on bologna, for although the numbers of *S. typhimurium* recovered dropped over storage for all samples, the rate of reduction on gel-treated samples was greater than on gel-control samples, which was, in turn, greater than on control samples.

The surface application of a 7% gelatin gel containing antimicrobials at 25.5 g/liter is equivalent in total antimicrobial supplied to the product as would be the case when 450 mg/kg is directly added to the sausage batter. Since the results obtained with 25.5 g/liter of antimicrobial were superior to the addition of 500 mg/kg to the batter, it appears that immobilizing the antimicrobial at the surface of the product is more effective than direct addition to the meat formulation.

Coating the surface of ham or bologna with a 7% gelatin gel without antimicrobials did not result in significantly different numbers of recovered colonies relative to control samples, except the in cases of *S. typhimurium* (Tables 6.11 and 6.12) and *E. coli* on ham (Table 6.3). The restriction on the growth of these organisms by the presence of the gelatin gel may have been caused by reduced access to nutrients, although the gelatin film had no effect on the other organisms studied.

The only organism for which significantly higher numbers of colonies were found on gel-treated samples, when plated on SMA as opposed to selective media was *B. thermosphacta*, indicating that for the other organisms tested the antimicrobial gel treatment did not allow growth of another opportunistic organism to dominate the product flora.

The observed increase in the effectiveness in the antimicrobial treatment against *B. thermosphacta, Lb. sakei, Lc. mesenteroides, L. monocytogenes* and *S. typhimurium* when applied as a gel surface treatment as opposed to direct addition to the sausage batter may have been due to a number of factors, in addition to localization of the antimicrobials at the site of bacterial growth at the surface. Padgett et al. (1998), observed that the inhibition zones of *Lb. plantarum* generated by soy protein and corn zein films in which lysozyme or nisin, or lysozyme with EDTA, or nisin with EDTA had been incorporated, were smaller when the films were heat-pressed as opposed to cast, indicating that heating reduced the antimicrobial activity. The direct addition of the antimicrobials to sausage batter may result in lowered activity when the product is cooked. The thermal stability of lysozyme is highly dependent upon the chemical environment within which heating occurs. The stability of lysozyme is high at low pH, in high salt or sugar solutions, but may be greatly reduced in the presence of proteins due to reaction with sulfhydryl groups (Proctor and Cunningham, 1988). Nisin thermal stability is also dependent on the chemical environment, with various food components having the potential to increase or decrease its thermal stability (Delves-Broughton et al., 1996).

Rose et al. (1999) suggested that nisin inactivation may occur in fresh meat by interaction of nisin with glutathione, catalyzed by glutathione-S-transferase; a reaction that could occur when mixed in product prior to cooking. However, this reaction can not be the sole cause of nisin inactivation in meat, as the activity of nisin was reduced over time at 4 °C in cooked pork (Fang and Lin 1995). It is possible that the immobilization of antimicrobials in the gelatin gel allowed diffusion of the antimicrobials over time, while reducing exposure

to inactivating agents in the meat. If this hypothesis is correct, then this antimicrobial gel system may be applicable for development and use with fresh meat.

Future work on this treatment system should focus on the development of packaging techniques that could be used to apply this treatment to commercial products. Work should also be done to clarify the effect of heat treatment of the product upon the activity of the antimicrobials.

Table 6.1. Growth (log CFU/cm^2) of *Brochothrix thermosphacta* on ham: without treatment (control); coated in a 7% gelatin gel (gel-control), or 7% gelatin gel containing 25.5 g/litre lysozyme:nisin::1:3 and 25.5 g/litre EDTA (gel-treated).

Recovery medium	Week 0		Week 1		Week 2		Week 3		Week 4	
and treatment	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std
STAA control	4.04^a	0.09	8.37^a	0.08	8.15^a	0.20	6.85^a	0.63	7.19^a	0.34
STAA gel-control	3.35^a	0.21	8.10^a	0.31	8.25^a	0.06	7.94^b	0.14	7.65^a	0.12
STAA gel-treated	nd^b	--	nd^c	--	nd^b	--	nd^c	--	nd^b	--
SMA control	4.30^a	0.07	8.12^a	0.14	8.27^a	0.09	7.90^b	0.06	7.66^a	0.32
SMA gel-control	3.86^a	0.10	7.81^a	0.46	8.23^a	0.04	8.15^b	0.12	7.87^a	0.20
SMA gel-treated	nd^b	--	4.32^b	1.04	7.73^a	0.29	7.39^{ab}	0.57	8.00^a	0.18

Differing superscripts in the same column indicate a significant difference among means, ($\bar{x}$), of 3 replicates plated in duplicate. Standard deviation (std).
nd - none detected.

Table 6.2. Growth (log CFU/cm^2) of *Brochothrix thermosphacta* on bologna: without treatment (control); coated in a 7% gelatin gel (gel-control), or 7% gelatin gel containing 25.5 g/litre lysozyme:nisin::1:3 and 25.5 g/litre EDTA (gel-treated).

Recovery medium	Week 0		Week 1		Week 2		Week 3		Week 4	
and treatment	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std
STAA control	3.16^a	0.20	8.01^a	0.07	8.11^a	0.06	7.91^a	0.10	7.77^a	0.07
STAA gel-control	3.15^a	0.12	7.80^a	0.20	7.95^a	0.05	7.77^a	0.06	7.66^a	0.06
STAA gel-treated	nd^b	--	nd^b	--	nd^b	--	nd^b	--	nd^b	--
SMA control	3.62^a	0.25	7.98^a	0.19	8.12^a	0.06	8.11^a	0.34	7.74^a	0.14
SMA gel-control	3.72^a	0.14	7.85^a	0.14	7.84^a	0.16	7.86^a	0.07	7.58^a	0.16
SMA gel-treated	nd^b	--	5.29^c	0.36	7.87^a	0.18	8.25^a	0.05	8.15^a	0.14

Differing superscripts in the same column indicate a significant difference among means, ($\bar{x}$), of 3 replicates plated in duplicate. Standard deviation (std).
nd - none detected.

Table 6.3. Growth (log CFU/cm^2) of *Escherichia coli* on ham:without treatment (control); coated in a 7% gelatin gel (gel-control), or 7% gelatin gel containing 25.5 g/litre lysozyme:nisin::1:3 and 25.5 g/litre EDTA (gel-treated).

Recovery medium	Week 0		Week 1		Week 2		Week 3		Week 4	
and treatment	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std
ctSMAC control	4.35^{a}	0.03	4.43^{a}	0.06	3.57^{ab}	0.05	3.73^{bc}	0.16	4.12^{bc}	0.44
ctSMAC gel-control	4.02^{a}	0.22	4.17^{a}	0.13	3.38^{b}	0.29	2.55^{d}	0.26	2.38^{d}	0.15
ctSMAC gel-treated	3.88^{a}	0.03	3.15^{b}	0.09	2.71^{b}	0.29	2.31^{d}	0.29	2.26^{d}	0.32
SMA control	4.77^{a}	0.35	4.64^{a}	0.05	4.78^{a}	1.15	4.88^{a}	0.94	6.37^{a}	1.19
SMA gel-control	3.94^{a}	0.25	4.27^{a}	0.07	3.78^{ab}	0.07	5.45^{ab}	1.62	5.17^{ab}	1.19
SMA gel-treated	3.95^{a}	0.26	3.78^{ab}	0.049	3.30^{b}	0.21	3.21^{cd}	0.12	3.16^{cd}	0.22

Differing superscripts in the same column indicate a significant difference among means, ($\bar{x}$), of 3 replicates plated in duplicate. Standard deviation (std).

Table 6.4. Growth (log CFU/cm^2) of *Escherichia coli* on bologna: without treatment (control); coated in a 7% gelatin gel (gel-control), or 7% gelatin gel containing 25.5 g/litre lysozyme:nisin::1:3 and 25.5 g/litre EDTA (gel-treated).

Recovery medium	Week 0		Week 1		Week 2		Week 3		Week 4	
and treatment	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std
ctSMAC control	4.17^a	0.07	3.27^a	0.19	2.89^c	0.12	2.14^c	0.25	2.38^c	0.57
ctSMAC gel-control	4.17^a	0.12	3.34^a	0.10	3.32^{bc}	0.32	2.63^c	0.43	2.20^c	0.34
ctSMAC gel-treated	4.04^a	0.09	3.07^a	0.06	2.59^c	0.11	2.31^c	0.38	2.23^c	0.32
SMA control	4.16^a	0.13	3.47^a	0.38	4.02^{ab}	0.48	5.76^b	0.58	6.51^a	0.25
SMA gel-control	4.09^a	0.17	3.59^a	0.11	4.60^a	0.25	6.97^a	0.75	5.47^b	0.61
SMA gel-treated	4.07^a	0.16	3.18^a	0.13	3.15^{bc}	0.06	3.13^c	0.13	2.89^c	0.15

Differing superscripts in the same column indicate a significant difference among means, ($\bar{x}$), of 3 replicates plated in duplicate. Standard deviation (std).

Table 6.5. Growth (log CFU/cm^2) of *Lactobacillus sakei* on ham: without treatment (control); coated in 7% gelatin gel (gel-control), or 7% gelatin gel containing 25.5 g/litre lysozyme:nisin::1:3 and 25.5 g/litre EDTA (gel-treated).

Recovery medium	Week 0		Week 1		Week 2		Week 3		Week 4	
and treatment	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std
MRS control	4.19^a	0.07	8.04^a	0.20	7.89^a	0.19	8.09^a	0.11	8.28^a	0.05
MRS gel-control	3.46^a	0.16	7.94^a	0.16	7.97^a	0.25	8.02^a	0.09	8.23^a	0.20
MRS gel-treated	nd^b	--	nd^b	--	0.63^b	1.41	4.33^b	0.11	4.23^b	0.17
SMA control	4.46^a	0.05	7.71^a	0.17	7.48^a	0.37	7.86^a	0.06	8.08^a	0.23
SMA gel-control	nd^b	--	nd^b	--	nd^b	--	3.48^b	0.54	3.73^b	0.34
SMA gel-treated	nd^b	--	nd^b	--	nd^b	--	nd^c	--	nd^c	--

Differing superscripts in the same column indicate a significant difference among means, ($\bar{x}$), of 3 replicates plated in duplicate. Standard deviation (std).
nd - none detected.

Table 6.6. Growth (log CFU/cm^2) of *Lactobacillus sakei* on bologna: without treatment (control); coated in a 7% gelatin gel (gel-control), or 7% gelatin gel containing 25.5 g/litre lysozyme:nisin::1:3 and 25.5 g/litre EDTA (gel-treated).

Recovery medium	Week 0		Week 1		Week 2		Week 3		Week 4	
and treatment	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std
MRS control	3.53^a	0.07	7.68^a	0.42	7.61^a	0.51	8.27^a	0.04	8.15^a	0.05
MRS gel-control	3.28^a	0.21	7.77^a	0.15	7.19^a	0.58	7.99^a	0.12	7.89^a	0.03
MRS gel-treated	nd^b	--	nd^b	--	nd^b	--	1.80^c	1.61	nd^c	--
SMA control	nd^b	--	nd^b	--	nd^b	--	4.23^b	0.24	4.19^b	0.22
SMA gel-control	nd^b	--	nd^b	--	nd^b	--	3.68^b	0.17	3.92^b	0.09
SMA gel-treated	nd^b	--	nd^b	--	nd^b	--	1.80^{bc}	2.54	nd^c	--

Differing superscripts in the same column indicate a significant difference among means, ($\bar{x}$), of 3 replicates plated in duplicate. Standard deviation (std).
nd - none detected.

Table 6.7. Growth (log CFU/cm^2) of *Leuconostoc mesenteroides* on ham: without treatment (control); coated in a 7% gelatin gel (gel-control), or 7% gelatin gel containing 25.5 g/litre lysozyme:nisin::1:3 and 25.5 g/litre EDTA (gel-treated).

Recovery medium	Week 0		Week 1		Week 2		Week 3		Week 4	
and treatment	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std
MRS control	4.63^a	0.14	7.74^a	0.09	7.66^a	0.06	7.10^a	0.30	7.79^a	0.29
MRS gel-control	3.93^a	0.16	7.80^a	0.05	7.74^a	0.08	7.74^a	0.02	7.61^a	0.08
MRS gel-treated	nd^b	--	nd^b	--	nd^b	--	1.30^b	0.93	nd^b	--
SMA control	4.69^a	0.09	7.67^a	0.11	7.61^a	0.08	6.89^a	0.25	7.17^a	0.20
SMA gel-control	4.09^a	0.08	7.75^a	0.05	7.60^a	0.06	7.12^a	0.73	7.16^a	0.22
SMA gel-treated	nd^b	--	2.05^b	2.09	nd^b	--	nd^c	--	nd^b	--

Differing superscripts in the same column indicate a significant difference among means, ($\bar{x}$), of 3 replicates plated in duplicate. Standard deviation (std).
nd - none detected.

Table 6.8. Growth (log CFU/cm^2) of *Leuconostoc mesenteroides* on bologna: without treatment (control); coated in a 7% gelatin gel (gel-control), or 7% gelatin gel containing 25.5 g/litre lysozyme:nisin::1:3 and 25.5 g/litre EDTA (gel-treated).

Recovery medium	Week 0		Week 1		Week 2		Week 3		Week 4	
and treatment	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std
MRS control	4.07^a	0.13	7.89^a	0.07	7.76^a	0.05	7.70^a	0.03	7.83^a	0.09
MRS gel-control	3.65^a	0.04	7.81^a	0.03	7.75^a	0.05	7.71^a	0.06	7.75^a	0.09
MRS gel-treated	nd^b	--	nd^b	--	nd^b	--	2.39^b	1.71	nd^b	--
SMA control	4.16^a	0.04	7.86^a	0.07	7.82^a	0.10	7.64^a	0.03	7.74^a	0.11
SMA gel-control	3.68^a	0.05	7.77^a	0.03	7.81^a	0.11	7.68^a	0.04	7.73^a	0.07
SMA gel-treated	nd^b	--	nd^b	--	nd^b	--	2.52^b	1.78	nd^b	--

Differing superscripts in the same column indicate a significant difference among means, ($\bar{x}$), of 3 replicates plated in duplicate. Standard deviation (std).
nd - none detected.

Table 6.9. Growth (log CFU/cm^2) of *Listeria monocytogenes* on ham: without treatment (control); coated in a 7% gelatin gel (gel-control), or 7% gelatin gel containing 25.5 g/litre lysozyme:nisin::1:3 and 25.5 g/litre EDTA (gel-treated).

Recovery medium	Week 0		Week 1		Week 2		Week 3		Week 4	
and treatment	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std
MOX control	5.11^a	0.27	8.20^a	0.09	8.35^a	0.12	8.58^a	0.07	8.25^a	0.09
MOX gel-control	4.70^a	0.17	8.18^a	0.45	8.45^a	0.17	8.53^a	0.07	8.31^a	0.18
MOX gel-treated	nd^b	--	nd^b	--	nd^b	--	2.33^b	1.66	0.65^b	0.93
SMA control	4.80^a	0.35	8.18^a	0.01	8.13^a	0.14	8.34^a	0.13	8.12^a	0.20
SMA gel-control	4.56^a	0.22	8.06^a	0.31	8.41^a	0.17	8.41^a	0.08	8.19^a	0.30
SMA gel-treated	nd^b	--	nd^b	--	nd^b	--	2.33^b	1.66	0.65^b	0.93

Differing superscripts in the same column indicate a significant difference among means, ($\bar{x}$), of 3 replicates plated in duplicate. Standard deviation (std).
nd - none detected.

Table 6.10. Growth (log CFU/cm^2) of *Listeria monocytogenes* on bologna: without treatment (control); coated in a 7% gelatin gel (gel-control), or 7% gelatin gel containing 25.5 g/litre lysozyme:nisin::1:3 and 25.5 g/litre EDTA (gel-treated).

Recovery medium	Week 0		Week 1		Week 2		Week 3		Week 4	
and treatment	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std
MOX control	4.32^a	0.22	7.74^a	0.31	7.95^a	0.15	8.30^a	0.17	8.13^a	0.08
MOX gel-control	3.99^a	0.24	7.63^a	0.15	8.26^a	0.04	8.23^a	0.04	8.06^a	0.15
MOX gel-treated	nd^b	--	nd^b	--	nd^b	--	nd^b	--	0.68^b	0.97
SMA control	4.46^a	0.05	7.71^a	0.17	7.48^a	0.37	7.86^a	0.06	8.08^a	0.23
SMA gel-control	4.30^a	0.05	7.89^a	0.05	8.15^a	0.05	8.01^a	0.13	7.85^a	0.15
SMA gel-treated	nd^b	--	nd^b	--	nd^b	--	nd^b	--	0.60^b	0.85

Differing superscripts in the same column indicate a significant difference among means, ($\bar{x}$), of 3 replicates plated in duplicate. Standard deviation (std).
nd - none detected.

Table 6.11. Growth (log CFU/cm^2) of *Salmonella typhimurium* on ham: without treatment (control); coated in a 7% gelatin gel (gel-control), or 7% gelatin gel containing 25.5 g/litre lysozyme:nisin::1:3 and 25.5 g/litre EDTA (gel-treated).

Recovery medium	Week 0		Week 1		Week 2		Week 3		Week 4	
and treatment	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std
BGS control	4.80^a	0.03	5.58^a	0.14	4.14^a	0.06	5.63^a	0.62	5.61^a	0.32
BGS gel-control	4.04^{ab}	0.14	4.69^{ab}	0.08	2.90^a	1.39	2.66^c	0.26	4.25^b	0.48
BGS gel-treated	3.59^b	0.33	2.73^c	0.24	nd^b	--	0.55^d	0.79	nd^c	--
SMA control	4.75^a	0.06	5.62^a	0.20	3.05^a	1.41	6.63^a	0.46	5.70^a	0.27
SMA gel-control	4.12^{ab}	0.10	4.52^b	0.18	3.91^a	1.02	4.11^b	0.54	4.56^b	0.25
SMA gel-treated	3.81^{ab}	0.18	1.31^c	1.31	nd^b	--	1.05^d	0.75	nd^c	--

Differing superscripts in the same column indicate a significant difference among means, ($\bar{x}$), of 3 replicates plated in duplicate. Standard deviation (std).
nd - none detected.

Table 6.12. Growth (log CFU/cm^2) of *Salmonella typhimurium* on bologna:without treatment (control); coated in a 7% gelatin gel (gel-control), or 7% gelatin gel containing 25.5 g/litre lysozyme:nisin::1:3 and 25.5 g/litre EDTA (gel-treated).

Recovery medium	Week 0		Week 1		Week 2		Week 3		Week 4	
and treatment	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std
BGS control	4.20^a	0.16	4.33^a	0.08	2.15^a	1.04	1.98^b	0.20	1.49^c	0.70
BGS gel-control	3.60^a	0.24	4.25^a	0.22	2.22^a	0.25	nd^c	--	1.18^{bc}	0.87
BGS gel-treated	3.26^a	0.23	2.86^b	0.08	nd^b	--	0.75^c	0.75	0.55^{bc}	0.79
SMA control	4.20^a	0.08	3.50^a	0.60	0.45^b	1.01	4.66^a	0.42	6.00^a	0.55
SMA gel-control	3.49^a	0.22	3.52^a	0.50	0.42^b	0.93	4.78^a	0.90	5.47^a	0.41
SMA gel-treated	3.60^a	0.19	2.38^b	0.42	nd^b	--	0.80^c	0.81	0.25^b	0.56

Differing superscripts in the same column indicate a significant difference among means, ($\bar{x}$), of 3 replicates plated in duplicate. Standard deviation (std).
nd - none detected.

7. Conclusions

Comparison of the investigations outlined in Chapters 3 and 4 into the antimicrobial activity of lysozyme and nisin with other antimicrobials indicate the difficulties involved in assessing antimicrobials for use in foods by using broth systems. Though both of these sets of experiments were conducted in the same nutrient broth systems the differing experimental designs resulted in different, though not contradictory, observations of antimicrobial interactions. The factorial experimental design described in Chapter 4 provides more complete information, more rapidly, on interactions than the response surface method described in Chapter 3. The factorial experimental design is superior to other experimental designs reported in the literature as it allows clear interpretation of collected data to determine whether or not an antimicrobial effect is significant and if interactions are occurring. In the experiments described in section 4 the factorial design was used to interpret turbidity data after a fixed period of incubation, which provides information on the effect of the antimicrobials upon the final population levels of the bacteria. The factorial method could also be applied to impedence detection data, which would allow evaluation of the effect on growth rate.

The results of the broth study described in Chapter 3 indicated that interactions between lysozyme and EDTA or nisin and EDTA did not increase the antimicrobial effect of these agents against Gram negative organisms, as reported in buffer system studies by other authors. Since EDTA was shown to affect organisms in broth it would appear that its ability to disrupt membranes is not necessarily inhibited in nutrient broth. The failure to observe an

effect of nisin on EDTA-treated Gram negative cells in broth may be a consequence of cells in the nutrient rich broth being able to compensate for the loss of low molecular weight molecules by increasing their rate of metabolism (see Carneiro de Melo et al., 1996).

The results of the broth study in Chapter 4, though not supporting a role for lysozyme or nisin in control of Gram negative organisms did indicate that the combination of lysozyme, nisin and EDTA as well as the NaCl and nitrite present in cured meat products presents a broader antimicrobial spectrum than any of the agents alone or paired. Addition of these antimicrobials to ham and bologna batter was demonstrated to affect the growth of number of spoilage and pathogenic bacteria at 8 °C, though Gram negative bacteria remained generally resistant (Chapter 5). The effectiveness of the antimicrobial treatment when applied to cured meat systems was observed to be dramatically increased when applied as a gel-immobilized surface treatment (Chapter 6). This result supports the hypothesis that surface application allows delivery of antimicrobials at a higher local concentration at the site microbial growth. Additionally, gel immobilization may preserve nisin activity by protecting it from interaction with inhibitors in meat. It is my recommendation, in light of these results, that the future development of novel antimicrobial systems for use in meat systems be directed towards application by packaging or coating systems. This would have the advantage of increasing the effectiveness of the treatment, as well as permitting removal of the antimicrobial source prior to consumption, which would be highly desirable in terms of consumer and regulatory acceptance.

Though broth experiments (Chapters 3 and 4) indicate that lysozyme, alone or with

other agents, can affect the growth of *Brochothrix thermosphacta, Pediococcus acidilactici, Enterococcus faecalis, Weissella viridescens* and *Staphylococcus aureus*, all of these organisms were observed to be sensitive to nisin to a greater or equal extent. Though trials of the antimicrobial system on cured meat, to date, have incorporated both nisin and lysozyme it has not been determined whether both these agents contribute significantly to the control of Gram positive bacteria. Comparison of trials on cured meat incorporating lysozyme with EDTA or nisin with EDTA would allow resolution of this question.

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