USE OF NATURAL ANTIMICROBIALS TO EXTEND THE SHELF-LIFE OF VACUUM-PACKAGED COOKED CURED MEATS

By

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Food Science
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This thesis is dedicated to my mother and father for believing and encouraging me to pursue for my goals.

Abstract

Lactic acid bacteria are found in vacuum-packaged cooked cured meats and dominate in the spoilage process (10^7-10^9 CFU·cm^{-2}) which leads to undesirable products. There is an increasing demand from both consumers and industry to have meat products with an extended shelf-life (>70 d) using natural preservatives. For the consumer, it means increasing the useful storage period (best before dates) of vacuum-packaged refrigerated cooked cured meat products without the need for increased concentrations of food additives. For industry, it means that these products can gain access to a wider market, possibly even Europe and Japan.

Typical spoilage microorganisms, *Leuconostoc mesenteroides* ssp. *mesenteroides*, *Lactobacillus sakei* and *Lactobacillus curvatus*, were subjected to a variety of preliminary testing against natural antimicrobial agents derived from spices (eugenol, allyl isothiocyanate), an organic acid (potassium lactate), a fatty acid (monolaurin), and lactic acid bacterial fermentations (bacteriocins – Alta® 2341 and Alta Mate®). The inhibitory activity of these agents was tested in mixtures which included an aqueous extract of wood smoke (liquid smoke) and a variety of phosphates and phytic acid as metal ion chelators. Individual inhibitors and their combinations were tested against bacteria in broth and agar diffusion tests where a variety of formats were used. Some of the tests used included monitoring changes in viable bacterial numbers or optical density in broth as well as formation of inhibition zones around discs or wells made in agar inoculated with test organisms. As the work progressed, *Lb. curvatus* was eliminated

from testing because all three organisms showed similar sensitivity profiles, but the two bacteria retained for further study demonstrated greater overall resistance.

The most successful agar diffusion procedure was found to be the Spiral Gradient Endpoint (SGE) Antimicrobial Susceptibility test where the target microorganisms were challenged with a wide range of antimicrobial concentrations. Results indicated that combinations of 350, 400 and 1000 ppm (w/v) eugenol, 0.5% and 1% (w/v) Alta Mate®, 0.4% (w/v) disodium pyrophosphate, and 3% (w/v) potassium lactate were able to inhibit these microorganisms at both 8°C and 25°C for 7 and 2 days, respectively. Therefore, these combinations were tested in meat model systems consisting of bologna and ham sausages.

All inhibitory systems used in vacuum-packaged bologna and ham sausages failed to inhibit both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* past 70 days at 8°C. In one meat trial with combinations of 350 or 400 ppm (w/w) eugenol, 0.5% (w/w) Alta Mate®, 0.4% (w/w) disodium pyrophosphate and 3% (w/w) potassium lactate, both bologna and ham sausages inoculated with the target microorganisms spoiled by 5 weeks, but the uninoculated sausages had a shelf-life >5 weeks at 8°C. The second meat trial yielded slightly better results when combinations of 1000 ppm (w/w) eugenol, 1% (w/w) Alta Mate®, 0.4% (w/w) disodium pyrophosphate and 3% (w/w) potassium lactate were used. While the inoculated bologna and ham sausages spoiled after 9 weeks, the inhibitor-treated, uninoculated sausages did not spoil until week 13. The untreated, uninoculated sausages spoiled about 2 weeks earlier. The water activity was significantly altered when the antimicrobials were incorporated into the bologna and ham emulsions, particularly when ham was treated with 3% (w/w) potassium lactate. The pH declined as

the products grew closer to being spoiled, which was expected. Colour was significantly affected in the presence of various combinations of antimicrobial agents incorporated into bologna and ham samples. Generally, bologna samples treated with antimicrobial agents were darker or had no change in colour. Ham samples treated with various combinations of antimicrobials were lighter in colour, but this may have been more related to mechanical/physical differences due to extra chopping of treated ham. Both vacuum-packaged bologna and ham were visually acceptable even when odour increased with increasing shelf-life.

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CHAPTER 1

INTRODUCTION

Vacuum-packaged ready-to-eat bologna and ham are popular cooked cured meat products offered for sale at the retail level because of their long shelf-life (>49 d). After these products are cooked, some are sliced prior to vacuum-repackaging. This handling process is believed to influence the bacterial level present and subsequently affects the shelf-life of the meat (Holley, 1997b). Shelf-life depends on the type and level of microorganisms present and their ability to grow (Holley 1997b; Mol et al., 1971). Lactic acid bacteria (LAB) are present in the microbial population found on vacuum-packaged cooked cured meat products (Mole et al., 1971; Holley, 1997a and 1997b; Dykes et al., 1995; Yang and Ray, 1994a; Egan, 1983) and eventually dominate, because they can grow at low temperatures under anaerobic conditions in the presence of 3-5% sodium chloride (NaCl) (Mol et al., 1971; Schillinger and Lücke, 1987a; Egan, 1983). As well they can survive in low pH conditions (Schillinger and Lücke, 1987a; Egan, 1983) and are insensitive to nitrites (Korkeala et al., 1992; Egan, 1983). Both homofermentative lactobacilli and leuconostocs are two types of LAB that are responsible for spoiling vacuum-packaged cooked cured meat products (Holley, 1997b; Dykes et al., 1995; Korkeala and Björkroth, 1997; Yang and Ray, 1994a) leading to dissatisfied consumers and economic loss to industry. As the level of LAB reach $>10^7$-10^9 colony forming units (CFU)$\cdot g^{-1}$ the shelf-life of the product is reached and products can be characterised as having off-odours, sour flavours, presence of cloudy purge, and

visually detectable slime within the package (Holley, 1997b; Korkeala and Björkroth, 1997; Korkeala et al., 1990; Yang and Ray, 1994a; Egan, 1983).

Natural antimicrobial agents are an important class of food additives (Davidson and Branen, 1993) and are becoming increasingly popular in the food industry, which views these antimicrobials as alternatives to the use of chemical preservatives. Presently, research has shown that a single compound is unable to be effective against all microorganisms in all storage conditions and in all foods (Davidson and Branen, 1993). Therefore, research has focused on combinations of antimicrobials that are effective against different groups of microorganisms, especially spoilage microorganisms such as LAB. Antimicrobials can be effective in preserving cooked cured meat products by either controlling the overall growth of the microorganisms or by killing the microorganisms. Generally, their mode of action falls into one of three categories: 1) reaction with the cell membrane, causing increased permeability and loss of cellular constituents; 2) inactivation of essential enzymes; or 3) destruction or functional inactivation of genetic material (Branen, 1993).

The objective of this research was two fold. First, to determine a variety of antimicrobials alone or in combination that effectively inhibits *Leuconostoc mesenteroides* ssp. *mesenteroides*, *Lactobacillus curvatus* and *Lactobacillus sakei* in MRS medium at 8°C and 25°C (*in vitro*). Finally, to determine if the antimicrobials have the ability to extend the shelf-life of cooked cured bologna and ham sausages at 8°C, by inhibiting spoilage microorganisms (*in vivo*).

CHAPTER 2

LITERATURE REVIEW

2.1. Vacuum-packaged cooked cured meat products

2.1.1. Definition

Vacuum-packaged meat is defined as meat that is placed in plastic bags or pouches followed by the removal of air using a vacuum packaging machine and the closing the bag or pouch with a heat-sealer. Low oxygen (O_2) permeable plastic films primarily exclude air and oxygen; packaging inhibits some of the spoilage microorganisms, and extends the refrigerated shelf-life of the meat product. The flexible film creates a barrier between the outside and inside of the package yielding slight differences in pressure. This creates an anaerobic environment where the contact between the meat and the plastic film is 'airless'. (Lundquist, 1987; Gill and Molin, 1991; Jay, 1992). Immediately after packaging, there is a small amount of residual O_2 present between the plastic film and the meat. The O_2 residue is consumed by the microbial flora and the meat itself, which results in an increased level of carbon dioxide (CO_2) during the first few days of storage. The quantities of O_2 and CO_2 are controlled mainly by the degree to which the plastic bag or pouch hinders the flow of these gases (Jay, 1992). The CO_2 causes aerobic microorganisms to be inhibited and anaerobic microorganisms to become the dominant microflora. (Jay, 1992; Gill and Molin, 1991). Mol et al. (1971) stated that better O_2 barrier films that create low-O_2 atmospheres increase the shelf-life of vacuum-packaged cooked meat. Overall, vacuum-packaged meat products are generally regarded as safe and virtually free from pathogenic microorganisms provided

recontamination does not occur at slicing and packaging (Jay, 1992; Russell and Gould, 1991).

2.1.2. Microbiology characteristics

Meat may contain a variety of microorganisms, depending on the type of meat and where it was processed. Meat contains a natural microflora that is present at the time the animal is slaughtered. When edible meat is stored during distribution before consumption, spoilage microorganisms dominate and generate metabolic end products, which usually lead to spoilage of these products (Korkeala and Björkroth, 1997).

2.1.2.1. Natural microflora

Initially, both mesophilic (cold intolerant) and psychrotrophic (cold tolerant) microorganisms are found in meat products. However, when the product atmosphere is altered through vacuum-packaging and stored at refrigerated temperatures, there is a shift in the microflora and psychrotrophic microorganisms become the dominant part of the microflora. There are different mixtures of psychrotrophic bacteria found on meat products, which are largely dependent upon the history of specific products. Examples of bacteria found on cured meat products are: *Carnobacterium* spp., *Brochothrix thermosphacta*, *Enterobacteriaceae*, and homofermentative and heterofermentative lactic acid bacteria (LAB) (Yang and Ray, 1994a; Jay, 1992; Leisner, et al., 1995; Borch et al., 1996).

2.1.2.2. Spoilage microflora

Spoilage microorganisms are found in the mixture of the natural microflora on surfaces of meat products. Initially, these spoilage microorganisms are not generally predominant, but become dominant during the long-term shelf-life of vacuum-packaged cured meat products (>49 d). Some dominant microorganisms found in cured meats are *Staphylococcus*, *Micrococcus*, *Lactobacillus*, *Microbacterium*, *Pediococcus*, *Streptococcus*, *Clostridium* and *Bacillus* species or their combinations (Cassens, 1994b). Other types of organisms can also become dominant, depending primarily on the nature of contaminants at packaging, the packaging film, the thermal process and the storage temperature used. Generally, it is the heterofermentative and homofermentative LAB that result in spoiling vacuum-packaged cured meat products (Gill and Greer, 1993; Borch et al., 1996; Korkeala and Björkroth, 1997; von Holy et al., 1992; Holley and McKellar, 1996; Leisner et al., 1995; Jay, 1992).

2.1.3. Shelf-life

Shelf-life is the period of time during which a particular meat product is stored prior to reaching the point of spoilage, or is the period of time from packaging until consumers find the product unacceptable. Shelf-life usually ends when microorganisms spoil the product, although physiochemical changes can sometimes precede microbial spoilage.

There are many opinions by food researchers concerning which key components extend the shelf-life of meats. Podolak et al. (1996) stated that shelf-life depends on packaging material, initial microbial contamination, storage temperature of the product,

and whether good manufacturing practices are carried out at the industrial level. Other researchers believe that pasteurisation temperatures will extend the shelf-life of cured meat products (Bell and Gill, 1982; Chyr et al., 1980; Brewer et al., 1992). Some research groups found that vacuum packaging will increase the shelf-life of a variety of meat products (Brewer et al., 1991; Gill and Jones, 1994; Holley et al., 1996a and 1996b; Kotzekidou and Bloukas, 1996).

As mentioned previously, vacuum-packaging inhibits certain types of microorganisms, primarily the aerobic microflora allowing only the anaerobic microflora to survive. Some of the anaerobic microflora actually help to extend the long-term storage period by inhibiting harmful members of the microflora found on the surface of the meat (Holley, 1997a; Leisner et al., 1995).

There is some evidence that a variety of bactericidal and bacteriostatic agents, including organic acids and their salts when added as ingredients into cured meat products, can extend product shelf-life (Papadopoulos et al., 1991a; Brewer et al., 1991; Podolak, et al., 1996; Brewer et al., 1992; Yang and Ray, 1994a). However, use of any of these agents must be accompanied by analyses that examine both changes in microbial flora, as well as sensory characteristics. Sensory acceptability plays a major role in consumer decisions and, therefore, at purchase, odour, colour, and general appearance should be unaltered from an acceptable range (Brewer et al., 1992).

2.1.4. Physical and chemical characteristics

2.1.4.1. Packaging

Packaging is an important step in providing good quality cooked cured meat products. The packaging material should be of low O_2 permeability and be a good barrier to moisture (Cassens, 1994c; Gill and Molin, 1991; Korkeala and Björkroth, 1997; Borch et al., 1996). Acceptable packaging films are made up of several layers of either flexible or more rigid plastic materials that allow a minimal quantity of gases to permeate through (Cassens, 1994c; Gill and Molin, 1991). Gill and Molin (1991) pointed out that the effectiveness of packaging films will differ due to: the type of plastic used, the proportion of residual gases and humidity present, the thickness of the plastic layers, and the temperature of storage. As well, these packaging materials should be transparent so that the product is visible to the consumer (Cassens, 1994c).

Desirable packaging films are made from food-compatible materials. There are many varieties of plastic materials used for vacuum packaging, but the best contain an O_2 barrier layer. Most popular high O_2-barrier film materials are laminated. These films can contain a gas-tight polyamide layer with a heat sealable polyethylene plastic layer (Korkeala and Björkroth, 1997). Another high-barrier packaging film was used by Holley (1997a). He found that using vacuum-packaging material consisting of a combination of ethylene vinyl alcohol, polyethylene and nylon film reduced the spoilage of ham and bologna type products. These two types of O_2-barrier film are examples of packaging materials that can be used.

Suitable packaging material can reduce colour degradation, oxidative rancidity and off-flavours associated with spoilage, and in some cases limit the growth of

pathogenic and spoilage microorganisms. Oxygen barrier films stabilise the nitrosylmyoglobin colour in meat, reduce the rate of oxidative rancidity associated with spoilage, and with the addition of some antimicrobial agents such as salt and nitrite, reduce the rate of growth by the microflora present (Cassens, 1994c; Jay, 1992; Ouattara et al., 1997; Borch et al., 1996; Yang and Ray, 1994a; Holley et al., 1996b; Stillmunkes et al., 1993).

The O_2 permeabilities are measured as the oxygen transmission rate (OTR) of a packaging material. The OTR of vacuum packages have a great influence on the shelf-life of the product (Holley et al., 1996a). Generally, films with O_2 permeabilities at ≤ 100 $cm^3/(m^2 \cdot 24\ h) \cdot atm$ are used for vacuum packaging. Gill and Molin (1991) believed that lower O_2 permeabilities would not greatly enhance the shelf-life of fresh meat, unless the oxygen transmission rate were <2 $cm^3/(m^2 \cdot 24\ h) \cdot atm$. However, others have found that films consisting of low O_2 permeabilities of 77 $cm^3/(m^2 \cdot 24\ h) \cdot atm$ were able to reduce aerobic microorganisms in cooked ham products (Kotzekidou and Bloukas, 1996). Packaging films with a low oxygen transmission rate of ≤ 15 $cm^3/(m^2 \cdot 24\ h) \cdot atm$ were considered best for optimal storage of cooked cured meat products provided proper refrigeration conditions were used (Holley et al., 1996).

2.1.4.2. Temperature

Storage temperature is a very critical issue. Generally, spoilage microorganisms grow between –3°C to 15°C. However, the growth rate of these psychrotrophic microorganisms is greatly reduced as the temperature decreases (Gill and Molin, 1991; Schillinger and Lücke, 1987a and 1987b; Gill and Newton, 1978; Holley et al., 1996b;

Borch et al., 1996). In other words, the lower the temperature used for storage of vacuum-packaged meats, the greater the shelf-life (Gill and Molin, 1991; Holley and McKellar, 1996; Borch et al., 1996). Optimal storage life is achieved at -1.5 ± 0.5°C, which is just above the temperature at which meat freezes (Borch et al., 1996; Cassens, 1994b). In commercial practice, cooked cured meats are stored at 4 to 9°C with a target of 4°C.

2.1.4.3. General product formulation

Microbial stability in cooked cured meat products is due to a number of essential factors, namely: salt, nitrite, sugar, pH, phosphates, and water activity (a_w).

Salts have been used for many years in brines or curing solutions. Salt, or sodium chloride (NaCl), is generally added: to slow or prevent the growth of microorganisms, reduce water activity, solubilize the myrofibrillar proteins in emulsion type cured meats, and provide flavour. The amount of NaCl used in cured meats depends on the desired character of the final product. Generally, the concentration of NaCl present is between 2.5 and 5% of the finished product (Mol et al., 1971; Hand et al., 1982; Blom et al., 1997; Maca, et al., 1997; Borch et al., 1996; Roberts et al., 1991; Cassens, 1994d; Frazier and Westhoff, 1988b).

Nitrite is responsible for the pink colour of cooked cured meats. As nitrite reacts with the myoglobin in the meat, the formation of nitric oxide myoglobin occurs. The red colour found in cured meat is better known as nitrosomyoglobin (Roberts et al., 1991; Cassens, 1994d; Borch et al., 1996; Cassens et al., 1979; Kono et al., 1994; Maca et al., 1997b; Frazier and Westhoff, 1988b). Upon heating of the meat, the pigment is

converted to dinitrosylhemochrome, yielding the characteristic pink colour of cured meats (Cassens, 1994d). Nitrite is an antimicrobial agent that is added to reduce the growth of food-poisoning bacteria in cured meats, but not spoilage microorganisms such as LAB. It is added to provide flavour but can have an oxidant effect. Sodium ascorbate is added to provide some anti-oxidative activity (Roberts et al., 1991; Cassens, 1994d; Borch et al., 1996; Cassens, et al., 1979; Nielsen, 1983; Kono et al., 1994; Maca et al., 1997b).

Sugar is incorporated into curing formulations primarily for flavour, particularly when high salt concentrations are used which can result in harsh flavour development (Cassens, 1994d). Sucrose can exert its preserving effect in the same manner as salt. One of the main differences is in relative concentrations, meaning that it requires about six times more sucrose than NaCl to effect the same degree of inhibition. The preserving effect of high concentrations of sugar, like salt, makes water unavailable to microorganisms (Jay, 1992). However, the amount of sugar normally used has little effect on preserving cured meat products (Cassens, 1994d).

A typical pH range for cooked cured meats is between 6 and 6.5 (Kotzekidou and Blukas, 1996; Korkeala and Björkroth, 1997; Holley et al., 1996b; Boerema et al., 1993; Mol et al., 1971; Blom et al., 1997; Borch et al., 1996). However, Holley et al. (1996b) found that ham and bologna had a slightly higher pH level of 6.52 and 6.58, respectively. In another study conducted by Holley (1997a) it was found that bologna and ham had an initial pH level of 6.86 and 6.65, respectively, which decreased as storage increased. This slight difference in pH is largely due to formulation differences.

Phosphates are used in the food industry as additives and generally: improve water binding and prevent moisture loss, delay onset of oxidative rancidity, and inhibit some microorganisms (Roberts et al., 1991; Flores et al., 1996; Cassens, 1994d; Lee et al., 1994). Other researchers have found that phosphates do not inhibit microorganisms, but rather increase their growth rate (Molin et al., 1984; Marcy et al., 1988; Choi et al., 1987; Lee et al., 1994). This difference is related to the ultimate pH, which develops following their use and is a function of their dissociation in aqueous environments. There are many types of phosphates that can be used in cooked cured meats. Their choice depends on the final product characteristics desired (Roberts et al., 1991; Flores et al., 1996; Cassens, 1994d). For example, sodium acid pyrophosphate is commonly used to assist in rapid development of cured meat colour (Cassens, 1994d).

Water activity (a_w) is a measure of unbound water present in an aqueous mixture compared with a pure solution of water (Cassens, 1994b). For cooked cured meats, the a_w is generally 0.97 or higher. If the water activity is higher, the chances of spoilage are greater (Holley et al., 1996a and 1996b; Borch et al., 1996; Kotzekidou and Bloukas, 1996; Chen and Shelef, 1992; Blom et al., 1997). Every type of bacteria has a certain a_w range within which they are able to grow. The growth rate and the lag phase of LAB are influenced by reduced a_w values. For example, a decrease in a_w value from 0.98 to 0.96 in bologna-type sausages results in a three-fold increase in lag time and a two-fold decrease in the growth rate of LAB (Borch et al., 1996).

2.2. Spoilage of vacuum-packaged cooked cured meat products

2.2.1. Definition

Spoilage of vacuum-packaged cooked cured meat products is a natural process due to biochemical and biophysical changes that occur in the meat over time, and this can be accelerated by the growth of microorganisms (Cassens, 1994b). Technically, an acceptable maximum bacterial level can define aerobic spoilage, but spoilage is more difficult to define this way in anaerobically packaged meats. The development of unacceptable odours and off-flavours, or the presence of unacceptable appearance are usually used to define the end of product shelf-life (Borch et al., 1996; Cassens, 1994b; Yang and Ray, 1994a).

2.2.2. Spoilage characteristics

There are certain characteristics that are associated with the spoilage of meat. They include: a shift in microflora to a monoculture or reduced microbial diversity, a reduction in pH, gas production within the vacuum-packaged meat, off-odours and off-flavours, or discolouration.

2.2.2.1. Shift in microflora

Initially, bacterial numbers on cooked cured meats are barely detectable, and this includes microorganisms capable of causing spoilage (Borch et al., 1996). At first, vacuum-packaged meats contain mixtures of aerobic and anaerobic bacteria introduced after cooking. As oxygen is depleted within the package, the aerobic microflora is inhibited while the anaerobic microflora flourishes. Spoilage of vacuum-packaged

cooked cured refrigerated meats occurs somewhere between 10^7 and 10^9 cfu·cm^{-2} or gram (Borch et al., 1996; Korkeala and Björkroth, 1997).

The main bacteria associated with spoilage of refrigerated vacuum-packaged cooked cured meat products are LAB, namely *Lactobacillus (Lb.)* spp. and *Leuconostoc (Lc.)* spp. (Borch et al., 1996; Yang and Ray, 1994a; Korkeala and Björkroth, 1997; Björkroth and Korkeala, 1996). These microorganisms preferentially consume carbohydrates present such as glucose and upon their exhaustion attack proteins in the meat. Proteins are broken down via proteolysis, to peptides and amino acids. The peptides and amino acids are further converted to undesirable end products that are associated with spoiled meats (Cassens, 1994a).

2.2.2.2. Change in pH

The pH of cooked cured meat products (pH ~6-6.5) is not by itself inhibitory to spoilage organisms (Korkeala and Björkroth, 1997; Egan, 1983). Meat tissue is usually sufficiently buffered so that visual spoilage resulting from growth of bacteria (usually LAB) occurs before there is a measurable change in meat pH. Korkeala and Björkroth, (1997) stated low pH values (pH 4.6-5.5) are found only when vacuum-packaged cooked sausages are undesirably spoiled. They found that the LAB population had reached spoilage levels (>10^7 colony forming units (CFU)·g^{-1}) before the pH declined. Kotzekidou and Boukas (1996) also found increased microbial numbers in sliced vacuum-packaged cooked ham had no correlation with pH change. Nonetheless, during refrigerated storage of vacuum-packaged cooked cured meats, the pH will eventually

drop below 6 as a result of the production of lactic/acetic acids by LAB (Dykes et al., 1991).

2.2.2.3. Gas production

Gas (CO_2) is often found in ham-type products and is generated by heterofermentative LAB such as *Lc. mesenteroides* (Jay, 1992; Yang and Ray, 1994a; Mäkelä et al., 1992). However, gas production can be found in other types of vacuum-packaged products. The formation of gas is indicated by the looseness of the packaging film around the product or may be extensive and slightly pressurise the package. Gas production readily signifies spoilage (Korkeala and Björkroth, 1997).

2.2.2.4. Off-odours and off-flavours

Off-odours are clear indications that the meat product has spoiled and is generally associated with the growth of lactic acid bacteria (Borch et al., 1991; Dainty and Mackey, 1992; Cassens, 1994b). There are many types of off-odours one can encounter. A putrid odour, formed by *Enterobacteriaceae* and *Pseudomonas* spp., is from meat stored aerobically at abusive temperatures (Cassens, 1994b; Borch and Molin, 1989). A sweet, cheesy odour is generated from meat products vacuum-packaged in highly O_2-permeable film and stored at abusive temperatures, or meat products originally stored anaerobically followed by the product being open and exposed to the air. It is likely due to the presence of *Enterobacteriaceae*, *B. thermosphacta* and homofermentative *Lactobacillus* spp. (Borch and Molin, 1989).

Off-flavours are frequently denoted as sour. They are generally due to the LAB, which produce acids such as lactic acid, acetic acid and formic acid: the levels depending on genus, species and growth conditions (Cassens, 1994b; Jay, 1992; Borch and Molin, 1989; Borch et al., 1991). Souring takes place when the bacteria utilise lactose and other sugars found in meat products to produce acids (Jay, 1992).

Off-odours and off-flavours are also caused when the fat in the meat product is oxidised causing stale or rancid characteristics. This is more of a problem for those products containing a high amount of fat and stored at abusive temperatures (Cassens, 1994b).

2.2.2.5. Temperature abuse

Temperature abuse is a major cause of accelerated spoilage of cooked cured meat products. It is not uncommon for products to be stored at refrigeration temperatures above 4°C and one frequently finds commercial products being held between 8 and 10°C (Franz and von Holy, 1996). Temperature abuse can occur at several points along the supply chain from producer to final consumer, including meat processing, refrigerated transportation, or at the retail level.

2.2.2.6. Appearance

Discolouration is usually the first sign of spoilage. The colour change of cured meat, from pink to a brownish colour, can often be due to high numbers of microorganisms present or upon exposure to light or air. Oxidation of cured meat pigment to nitrosyl-metmyoglobin yields an undesirable brownish colour.

Catalase-negative LAB, such as leuconostocs, produce hydrogen peroxide (H_2O_2) while other LAB generate hydrogen sulfide (H_2S) as by-products, which in turn produce the 'green' colour seen on the surfaces of cured meats. 'Greening' can take place when the vacuum-packaged meat is exposed to oxygen. The O_2 acts as a hydrogen acceptor producing H_2O_2. When H_2O_2 is produced it reacts with dinitrosylhemochrome to produce green hydroperoxymetmyoglobin and eventually greenish oxidised porphyrins. These both give meat a 'green' appearance (Jay, 1992; Borch et al., 1996; Grant et al., 1988).

Vacuum-packaged cooked cured meat products may show signs of slime which can be produced by LAB and enterococci (Cassens, 1994c; Jay, 1992; Björkroth and Korkeala, 1996; Mäkelä et al., 1992). The slime is found on the surface of moist meat. While it does not indicate spoilage of the product in small amounts, it does contribute to product discolouration (Cassens, 1994c; Jay, 1992). Korkeala et al., (1988) stated that slime is an early indication of spoilage seen prior to the retail sell-by date.

2.2.3. Spoilage of cured meat products by LAB

It is well known throughout the literature that LAB spoil cooked cured meat products (Yang and Ray, 1994a; Korkeala and Björkroth, 1997; Gill and Greer, 1993; Holley, 1997a and 1997b; Dykes et al., 1995; Holley, 1996a). Lactic acid bacteria are Gram-positive, non-motile, non-sporing, non-acid-fast rods or cocci. They are facultative anaerobes that can survive in an O_2 atmosphere but prefer to live in an anaerobic environment (usually between 5 and 10% CO_2). These bacteria are also: catalase-negative, oxidase-negative and are often arginine-negative. They utilise sugars to

produce mainly lactic acid, but acetic acid, CO_2 and ethanol can also be produced. The primary substrates used for growth by LAB are glucose, or other simple sugars. They usually grow up to $10^8 \cdot cm^{-2}$ before spoilage occurs. LAB also metabolize the amino acids valine and leucine, which are degraded along with the production of volatile fatty acids to produce an acid-dairy flavour in the meat product (Gill and Greer, 1993; Condon, 1987; Frazier and Westhoff, 1988a; Grant et al., 1988). These microorganisms compete with others for dominance. They are able to inhibit other organisms such as the psychrotrophic Enterobacteriaceae, *B. thermosphacta*, *Salmonella* and *Staphylococcus aureus*, primarily by the lactic acid they produce, while at the same time competing for nutrients (Cassens, 1994b).

2.2.3.1. Homofermentative and heterofermentative LAB

LAB can be divided into two major groups, homofermentative and heterofermentative, based on their hexose fermentation pathways: homofermentative lactobacilli degrade hexose via glycolysis, whereas heterofermentative lactobacilli degrade hexose via the pentose-phosphate pathway. Kandler (1983) and Jay (1992) provided good diagrammatic presentations of the pathways the two groups of LAB use.

Homofermentative lactobacilli produce lactic acid through the fermentation of glucose. However, they can produce 2 moles of lactic acid from any fermentable hexose. They produce more acid than heterofermentative LAB (Gill and Greer, 1993; Kandler, 1983; McDonald, et al., 1987; Condon, 1987; Frazier and Westhoff, 1988a; Jay, 1992).

Heterofermentative lactobacilli produce a mixture of lactate, carbon dioxide and ethanol from glucose. They can produce acetic acid, lactic acid and CO_2 when fructose is

the only source of carbohydrate. As well, they can produce mannitol by reducing portions of fructose. Heterofermentative LAB are often the main cause of spoilage in vacuum-packed meat (Gill and Greer, 1993; Kandler, 1983; McDonald, et al., 1987; Condon, 1987; Frazier and Westhoff, 1988a; Jay, 1992).

In general, these LAB are able to survive in low pH environments compared to their competitors, the Gram-negative bacteria (Egan, 1983; Franz and von Holy, 1996).

2.2.3.1.1. *Leuconostoc mesenteroides* ssp. *mesenteroides*

Leuconostoc mesenteroides ssp. *mesenteroides* is a heterofermentative LAB. They exist naturally in milk, on plants, vegetables, grapes, as well as on raw and finished meat products (Yang and Ray, 1994a; Garvie, 1986; Frazier and Westhoff, 1988a). They are elliptical in shape and are usually found in pairs and short chains. They do not form spores and are usually 0.5-0.7 by 0.7-1.2 μm in size and yield smooth colonies greyish-white in colour. These LAB can grow over a wide temperature range between 10 and 37°C, but their optimum growth temperature is between 20 and 30°C. They cannot survive heating at 55°C for 30 min. Since they are heterofermentative LAB, glucose is degraded to produce 1 mole of D(-)-lactic acid, ethanol and CO_2 via the pentose pathway (Garvie, 1986). While they can grow at refrigeration temperatures they do so at a much slower rate. They are also non-pathogenic to animals and to humans.

These organisms require valine and glutamic amino acids for growth (Garvie, 1986; Frazier and Westhoff, 1988a). Yang and Ray (1994a) have suggested that most heterofermentative leuconostocs produce bacteriocins that may provide them with the advantage to grow, producing gas and other unacceptable spoilage characteristics found

in meats. *Leuconostoc* ssp. are more sensitive to NaCl and $NaNO_2$ compared with homofermentative LAB (Korkeala and Björkroth, 1997; Korkeala et al., 1992).

2.2.3.1.2. *Lactobacillus sakei* and *Lactobacillus curvatus*

Like the heterofermentative LAB, the homofermentative LAB are also predominant in spoiled meat products (Yang and Ray, 1994a). *Lactobacillus* spp. are rod-shaped, often form chains, particularly in the later phases of growth, and are non-sporing. They are Gram-positive, however, these organisms become Gram-negative when the pH is more acidic. Their optimum growth range is between 30 and 40°C (Rogosa, 1974) and they cannot normally grow at 45°C (Kandler and Weiss 1986). Although they can withstand acid conditions, the optimal pH is usually in the range of 5.5 and 5.8, or less. Like *Lc. mesenteroides* ssp. *mesenteroides*, lactobacilli colonies are greyish-white in colour and they are found in many environments such as dairy products, grains, fruits, beer, wine, and meat products. As well, they are normally non-pathogenic to animals or humans (Kandler and Weiss, 1986; Frazier and Westhoff, 1988a).

Two species of lactobacilli are frequently found on vacuum-packed cooked cured meats stored at refrigerated temperatures. These are *Lb. curvatus* and *Lb. sakei*. One difference between the two species of lactobacilli is that *Lb. curvatus* is more sensitive to NaCl and $NaNO_2$ than *Lb. sakei* (Korkeala and Björkroth, 1997). Another difference is that *Lb. curvatus* cells are more bean-shaped than *Lb. sakei*, and the former cells are also rod-shaped with a slight curve. Also, *Lb. curvatus* cells are smaller (0.7-0.9 x 1-2 μm) than those of *Lb. sakei* (0.6-0.8 x 2-3 μm) (Kandler and Weiss, 1986).

2.2.3.2. Selective and differential media

There are several selective and differential media that are used to distinguish among the different types of LAB. Selective media are used to suppress the growth of unwanted groups of bacteria, thereby allowing only the specific desired group to grow. Differential media allow distinction of one type of organism from another and may at the same time, repress unwanted groups of organisms. The latter media are used for the detection of shifts in the type of microorganisms present (Gill and Greer, 1993).

Selective media usually have incorporated substance(s) like antibiotics to inhibit most of the bacteria except the target microflora. There are other ways in which to select target microflora such as choices of atmospheres, pH of the medium, salt concentrations and incubation conditions (Gill and Greer, 1993).

Differential media usually distinguish target bacteria from others without inhibiting growth. A differential medium may involve use of a pH indicator, a chemical indicator(s), or substrate(s) that are characteristically used by the target microorganisms. This usually leads to a distinctive change in the appearance of the colonies; for example, the colour, or of the surrounding medium (Gill and Greer, 1993).

2.2.3.2.1. M5 medium

Originally, McDonald et al. (1987) developed a differential medium called HHD that distinguished between homofermentative and heterofermentative LAB. This medium was based on the increase in acid production by homofermentative lactobacilli from a fixed amount of fructose. It contained a pH indicator, which allowed observation of differences in pH by the resulting changes in colour. The LAB that were

homofermentative were perceived as blue colonies, whereas the heterofermentative LAB were perceived as white colonies.

The HHD medium worked well in most foods, however, some strains belonging to the lactobacilli family were unable to grow or grew poorly; for example, LAB strains found in wines and musts. Zúñiga et al. (1993) developed an alternative medium called M5, which was designed to maintain the strains of LAB found in wine. This highly specialized medium can distinguish between homofermentative and heterofermentative lactobacilli that utilize fructose as a carbon source. Like the HHD medium, M5 has a pH indicator called bromocresol green, which helps to differentiate between homofermentative and heterofermentative LAB. The M5 medium is not commercially made.

Zúñiga et al. (1993) altered HHD medium by removing casaminoacids, bactosoytone, glucose, and tomato juice, and by adding L-cysteine HCL, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, and calcium pantothenate to create the new M5 medium. Another difference is that M5 is adjusted to a final pH of 6.5.

For best results, M5 medium should be incubated anaerobically at 25°C with 100% CO_2 for up to 7 days depending on the strains. The anaerobic atmosphere is important because the LAB utilise the fructose as an electron acceptor, changing fructose to mannitol. This reduction is important in observing the proper results from all strains. Zúñiga et al. (1993) found that incubating M5 plates aerobically sometimes gave improper results because heterofermentative strains produce higher amounts of acid for two reasons: (1) an increased amount of fructose can be converted into acid and not reduced to mannitol and; (2) additional acetic acid can be produced (Condon, 1987).

When properly incubated, *Lb. sakei* formed blue colonies while *Lc. mesenteroides* ssp. *mesenteroides* remained white on the bluish coloured M5 medium.

2.2.3.2.2. All purpose tryptone (APT) medium

All purpose tryptone comes in broth or agar form, and is commercially available. For this work, both types were used interchangeably. The APT is used to cultivate almost all organisms present in meat. It is a nonselective medium (Atlas, 1995).

2.2.3.2.3. Violet Red Bile Glucose (VRBG) medium

Violet Red Bile Agar is used primarily for selecting coliform bacteria. It is made from commercially available VRB agar, has a final pH of 7.4 and is incubated aerobically between 35 and 37°C for 18-24 h. An overlay is usually required once the bottom layer has hardened and the sample has been inoculated and dried. This medium (VRB) allows coliforms to appear as red-purple colonies surrounded by a red-purple halo on the violet coloured agar (Gill and Greer, 1993; Anon, 1987b; Atlas, 1995).

One percent (w/v) glucose can be incorporated into VRB medium to allow enumeration of *Enterobacteriaceae* which includes some lactose negative strains. This modified medium containing 1% (w/v) glucose was termed VRBG medium (Mol et al., 1971).

2.2.3.2.4. Streptomycin thallous acetate actidione (STAA) medium

Originally, this medium was used to isolate and enumerate *Brochothrix thermosphacta* from meat products. This selective medium was later modified by the

additions of several antimicrobial agents, which exclude other microorganisms from meats, and allows *B. thermosphacta* to grow. The antimicrobial agents are streptomycin sulphate, actidione and thallous acetate (Gardner, 1966; Gill and Greer, 1993; Anon, 1987c).

The antimicrobial agents, also known as selective supplements, are filtered sterilised before they are added to the sterile medium. It has a pH of 7, and is incubated aerobically at 22-25°C for 48 h. The medium is a pale straw colour with *B. thermosphacta* appearing as white colonies 0.5 to 1 mm in diameter (Gill and Greer, 1993; Anon, 1987c; Atlas, 1995). STAA is not made commercially.

2.2.3.2.5. de Man, Rogosa and Sharpe (MRS) medium

The MRS medium was primarily developed to allow the growth of all lactobacilli and replace tomato juice medium (de Man, Rogosa and Sharpe, 1960; Anon, 1987a). MRS is not selective as pointed out by Egan (1983) because it contains magnesium and acetate, which allow the growth of lactobacilli, pediococci, leuconostocs and streptococci. However, MRS can become somewhat selective by lowering the pH to 5.7. Unfortunately, it inhibits growth of some of the meatborne lactobacilli, such as *Carnobacterium* (Gill and Greer, 1993; de Man, Rogosa and Sharpe, 1960). MRS allows most LAB to grow, although some strains grow at a slower rate than others (Egan, 1983).

MRS is commercially available and has a final pH between 6 and 6.5. The medium is amber in colour, where the lactobacilli appear as white or grayish colonies between 0.5 and 2.5 mm in diameter (Gill and Greer, 1993; de Man, Rogosa, Sharpe, 1960; Anon, 1987a; Atlas, 1995).

2.2.3.3. Factors effecting growth of LAB

2.2.3.3.1. pH

The pH of most foods has a minimal effect on LAB growth; they thrive on acidic conditions as low as pH 5-5.3. With the production of lactic acid by LAB, the pH decreases slightly during extended shelf-life of the product. Many other spoilage microorganisms are unable to grow in the presence of the lactate produced (Borch et al., 1996; Kotzekidou and Bloukas, 1996; Holley et al., 1996a, 1996b; Korkeala and Björkroth, 1997). Most often, there is little change in meat pH by the time meat is spoiled. Some researchers have found that LAB can survive in spoiled vienna sausages with a pH of 4.8 to 4.6 (von Holy et al., 1991).

2.2.3.3.2. A_w

As mentioned previously, water activity (a_w), does not normally decrease during the shelf-life of the product. Therefore, a_w does not affect the growth of LAB as long as the initial a_w is higher than 0.91, any lower and the LAB are inhibited. If the a_w is decreased beyond the optimum level at which LAB can grow, the length of their lag phase is increased, their growth rate and their final population size are decreased (Jay, 1992; Borch et al., 1996; Russell and Gould, 1991). For example, Borch et al. (1996) found that bologna-type sausage in which the a_w was decreased from 0.98 to 0.96 resulted in a two to three-fold increase in the lag time of LAB.

2.2.3.3.3. Nutrient requirements

For LAB to grow and function they must have elements such as water, a source of energy and nitrogen, vitamins and minerals.

Water is important for growth and well-being of LAB. As mentioned previously, if the a_w is below 0.91, the length of the lag phase of growth is increased and the growth rate and final population size is decreased (Jay, 1992; Borch et al., 1996; Russell and Gould, 1991). Water is very important to LAB as it affects all metabolic activities; meaning, all chemical reactions of the cells require an aqueous environment (Jay, 1992). Water activity is described in the previous section.

Lactic acid bacteria require carbohydrates, specifically glucose, as energy and carbon sources (Garvie, 1986; Kandler and Weiss, 1986). In the case of *Leuconostoc* spp., fructose is preferred as the carbohydrate source (Garvie, 1986).

Complex growth factors and amino acid (nitrogen source) requirements for growth are needed by LAB to function normally. These requirements differ between genus and species within LAB. For instance, *Leuconostoc* spp. require nicotinic acid, thiamine, biotin and either pantothenic acid or a pantothenic acid derivative. The subspecies, *Lc. mesenteroides* ssp. *mesenteroides* only requires glutamic acid and valine for growth (Garvie, 1986). *Lactobacillus* spp. require pantothenic acid and nicotinic acid, and thiamine is only necessary for the growth of heterofermentative lactobacilli.

Vitamins are not a necessary requirement for LAB to grow and function. However, some strains are vitamin-dependent and are used primarily for bioassays of vitamins. Essentially, LAB require peptone, yeast extract and a source of carbohydrate to grow and survive.

The requirements for essential nutrients are normally met when media such as MRS are used which contains fermentable carbohydrate, peptone, meat and yeast extract, manganese, Tween 80, and acetate (de Man et al., 1960).

2.2.3.3.4. Temperature

Low temperatures alone do not prevent the growth of LAB, even at 0°C (Korkeala and Björkroth, 1997; Russell and Gould, 1991), however, low temperatures have been shown to slow the growth of these spoilage organisms (Borch et al., 1996; Russell and Gould, 1991). For example, Korkeala et al. (1990) found that *Lb. sakei* was able to grow on MRS agar at temperatures below 0°C.

2.2.3.3.5. Oxygen requirement

Most LAB strains are able to tolerate O_2 to a certain degree; however, they are not the primary spoilage organisms on meat products in the presence of O_2. The LAB are replaced by *B. thermosphacta* or enterobacteria (Nielsen, 1983; Borch and Molin, 1989). Some LAB strains will be completely inhibited, while others will experience a slower growth rate in the presence of O_2 (Condon, 1987).

2.2.3.3.6. Others factors

Other factors that can inhibit the growth of LAB are combinations of NaCl and $NaNO_2$, and antimicrobials. The latter will be discussed in the next section.

Some researchers have found that combinations of NaCl and low concentrations of $NaNO_2$ have inhibitory effects on the growth of spoilage bacteria (Korkeala and

Björkroth, 1991; Korkeala et al., 1992). Korkeala et al. (1992) found that 0, 50, 100, 200 and 400 mg/l concentrations of $NaNO_2$ in the absence of NaCl had an inhibitory effect at 24 h on the growth of LAB strains isolated from vacuum-packaged cooked ring sausages. However, a more pronounced inhibitory effect was found with 400 mg/l of $NaNO_2$ compared to 200 mg/l. Zhang and Holley (1999) also found that the LAB strains tested showed no significant reduction in growth in modified MRS (mMRS) broth when exposed to 100, 150 or 200 ppm of $NaNO_2$. Korkeala et al. (1992) found that a 1-2% addition of NaCl enhanced the growth of LAB compared to 0% NaCl. As well, NaCl concentrations of 3-7% had an inhibitory effect on the growth of spoilage lactic acid bacteria tested. The NaCl results were consistent with the findings from Zaika et al. (1978) where growth of LAB was inhibited with increasing amounts of NaCl (from 1-7%) in fermented Lebanon bologna-type sausages. Zhang and Holley (1999) also detected inhibitory effects with LAB strains when 4% and 5.5% NaCl were present in the mMRS broth; even some strains were reduced by 1-2 log $CFU \cdot ml^{-1}$.

Korkeala et al. (1992) suggested that NaCl or $NaNO_2$, used separately, or in combination might selectively allow the LAB population to multiply in vacuum-packaged meat products. For total inhibition of LAB, considerable amounts of NaCl and $NaNO_2$ are needed (Korkeala et al., 1992). However, other researchers have found that both NaCl and $NaNO_2$ in high concentrations are able to inhibit the growth of LAB (Mol et al., 1971; Egan, 1983; Korkeala et al., 1992).

As mentioned previously, leuconostocs are more sensitive to NaCl and $NaNO_2$ than homofermentative lactobacilli.

2.3. Antimicrobial agents

2.3.1. Definition

Antimicrobial agents can be used as food preservatives by reducing the rate of biological deterioration and spoilage of food (Lück and Jager, 1997; Branen, 1993). Three different approaches involving physical, chemical and biological methods have been used to prevent meat decay. Physical methods include heating, cooling, and refrigeration. Chemical methods include the use of preservatives, which are chemical substances that inhibit the development of microorganisms or kill them (Lück and Jager, 1997). For example, common salt and vinegar are two preservatives used today to extend the shelf-life of many food products by inhibiting microorganisms. Biological methods include use of high purity, harmless bacterial cultures that have an inhibitory effect on undesirable spoilage microorganisms. When these cultures are added to the food they are known as "protective cultures" (Lück and Jager, 1997).

2.3.2. Antimicrobial action on microorganisms

When antimicrobial action is effective in controlling the overall growth of the microorganisms, it directly destroys all, or parts of the microorganisms (Lück and Jager, 1997; Branen, 1993). Lück and Jager (1997) stated that one or several types of actions can explain antimicrobial action. These occurrences have an influence on: DNA; protein synthesis; enzyme activity; the cell membrane; the cell wall, and transport mechanisms for nutrients.

The inhibitory effect on enzyme activity, or enzyme and protein synthesis in microorganisms was regarded as the most important factor in explaining antimicrobial

action (Lück and Jager, 1997). Recent speculation by some researchers infer that some antimicrobials act on the cell wall and membrane, which in turn affects their structure, energy status and the transport of nutrients into the cell (Eklund, 1980; Lück and Jager, 1997). This process involves lipophilic antimicrobials attacking and destroying the semi-permeable character of cell membrane. This increases the flow of protons to the cell, causing it to use more energy to keep its interior pH neutral (Lück and Jager, 1997; Freese et al., 1973).

The antimicrobial should have a certain degree of water solubility since the microorganisms grow in this phase. The agent should also be somewhat lipid soluble because it needs to be able to penetrate the hydrophobic areas of the cell membrane of the organism (Branen et al., 1980; Robach, 1980).

The ability of the antimicrobial to work effectively depends on: processing conditions, storage of the product, product composition, level and type of microorganisms, as well as the nature of the antimicrobial used (Branen, 1993). Further, some combinations of antimicrobials can interact and increase the antimicrobial range of action and potency, and thus lower the threshold concentrations of the individual antimicrobial compounds needed for inhibition (Lück and Jager, 1997; Branen, 1993).

2.3.3. Essential oils

2.3.3.1. Definition

Essential oils are a group of antimicrobial agents that are odorous in nature, soluble in alcohol, poorly soluble in water, and consist of a mixture of esters, aldehydes, ketones and terpenes. They are widely used in the food industry as flavourings, fragrant

substances and are active against microorganisms. Essential oils can be obtained from spices, herbs, berries, roots and stems of plants (Wilkins and Board, 1989).

2.3.3.2. Allyl isothiocyanate (AIT)

Allyl isothiocyanate (CH_2=CH-CH_2-N=C=S), is one of a group of sulfur-containing compounds found naturally in plants; for example, horseradish and black mustard. The AIT is only found when plant tissues are disrupted, either by enzymatic or mechanical means, and the cell-wall-bound myrosinase enzyme comes into contact with the glucosinolate, sinigrin. This catalyses the formation of the AIT which becomes part of the volatile essential oil (Lim and Tung, 1997; Delaquis and Sholber, 1997; Isshiki et al., 1992).

Allyl isothiocyanate is fat soluble, water insoluble, and is soluble in alcohol; for example, ethanol (Sekiyama et al., 1994; Delaquis and Sholberg, 1997; Lück and Jager, 1997). It is a very unstable compound and it can react with amino acids, or a protein to yield additional compounds. In addition, water decomposes it through nucleophilic attack (Ohta et al., 1995a; Kawakishi and Kaneko, 1987; Delaquis and Sholberg, 1997). However, the decomposition of AIT in aqueous solutions can be suppressed in the presence of dextrins and polysaccharides (Ohta et al., 1995b).

The AIT is used as a flavouring agent, a spice that is a component of the essential oil contributing strong antibacterial and antifungal activity (Lim and Tung, 1997; Sekiyama et al., 1994; Ohta et al., 1995a; Ohta et al., 1995b; Delaquis and Mazza, 1995). The antimicrobial activities of AIT are well known. However, the active ingredient involved has had little attention from the food industry. Some consumers now prefer

natural ingredients (for example AIT) to synthetic preservatives (for example salt and nitrite). Researchers have focused on antimicrobial agents from plants (Lim and Tung, 1997; Delaquis and Sholberg, 1997). They have found that AIT is able to inhibit a wide range of microorganisms on the surface of agar (Isshiki et al., 1992; Ward et al., 1998; Delaquis and Sholberg, 1997). For example, Isshiki et al. (1992) found that bacteria were inhibited by AIT at a range between 34 and 110 ng/ml. They suggested that AIT might be used as an antimicrobial vapour in modified or controlled atmosphere packaging (Delaquis and Sholberg, 1997; Lim and Tung, 1997). Delaquis and Sholberg (1997) discovered that microorganisms exposed to AIT experienced a delay in colony development, which led to a loss of viability in vegetative bacterial cells. They also found that fungi and Gram-negative aerobic bacteria were inhibited by lower concentrations of AIT than the facultative anaerobic foodborne pathogens.

Allyl isothiocyanate action inhibits specific carriers in the electron transport chain and acts as an uncoupler of oxidative phosphorylation. It has been mentioned that non-specific reactions with other key enzymes, or proteins may be required to achieve a significant antimicrobial effect. Also, increased AIT volatility and improved diffusion into the cell or spore may result from storage at elevated temperatures; higher than room temperature (Delaquis and Sholberg, 1997).

Allyl isothiocyanate is presently permitted in food in Japan as long as the compound is extracted from natural sources. It is also used in North America by addition of mustard to foods, for example, to mayonnaise to enhance the flavour (Delaquis and Sholberg, 1997). As well, AIT from mustard oil has been approved in Italy in the form of

paraffin wax tablets for the stabilization of wine in large containers (Lück and Jager, 1997).

From the research that has been done so far on AIT and its current use in Japanese foods, it was thought that this natural preservative might to be effective for inhibiting the target organisms used in this work and extend the shelf-life (>49 d) of either or both bologna and ham sausages.

2.3.3.3. Spices

For centuries, spices have been used for their preservative and medicinal powers, besides their flavour and odour qualities (Conner, 1993; Zaika, 1988). Only recently, have their antimicrobial properties become of interest. The antimicrobial properties reside in the essential oil of the spice and normally these oils are obtained through commercial distillation processes (Connor, 1993).

Spices can be obtained from a variety of dried aromatic plant products. For example, spices may come from leaves (rosemary, sage), flowers and flower buds (clove), bulbs (garlic, onion) and fruit (pepper, cardamom) (Shelef, 1983; Beuchat and Golden, 1989). Spices are used in many foods, ranging from breads and pickles to soft drinks and cured meats.

Spices affect all stages of microbial growth, for example, lag phase is extended, growth rate is decreased and the population is reduced (Shelef, 1983). It has been found that spices such as sage, rosemary and clove were able to effectively inhibit Gram-positive bacteria. Gram-negative bacteria were found not to be as sensitive as Gram-

positive bacteria to these spices (Wedorff and Wee, 1997; Wendakoon and Sakaguchi, 1995; Farag et al., 1989; Aureli et al., 1992; Shelef, 1983).

Spices may be part of the answer in extending the shelf-life of many foods, but their use is presently under scrutiny. Spices, especially ones that are not further processed, can contain high levels of microbes and need additional disinfecting before being used in foods (Lück and Jager, 1997). Ceylan et al. (1998) stated that from a food safety aspect, use of natural spices has potential application for producing safer food products.

2.3.4. Bacteriocins

2.3.4.1. Definition and description

Bacteriocins are ribosomally synthesized antimicrobial proteins or peptides formed by bacteria that inactivate other closely related bacteria (Lück and Jager, 1997; Lücke and Earnshaw, 1991; Chen and Montville, 1995). Bacteriocins can be used to preserve food either in their natural form or as protective cultures that produce bacteriocins in foods (Lück and Jager, 1997). There is a growing popularity of bacteriocins because they meet consumers' demands for natural substances to preserve foods (Lück and Jager, 1997; Hoover, 1993).

The most extensively studied bacteriocins are those from LAB. Many bacteriocins inhibit the growth of a variety of food-borne pathogens and spoilage organisms; for example, staphylococci, streptococci, *Listeria*, clostridia and bacilli (Lewus et al., 1991; Nettles and Barefoot, 1993). Bacteriocins produced by Gram-positive bacteria are not effective against Gram-negative bacteria such as salmonellae,

and the most common types have no effect on yeasts and fungi. Korkeala and Björkroth (1997) stated that bacteriocins can be inactivated by food constituents thereby making their use very product-category specific. Some bacteriocins are heat sensitive and can be used only in foods that undergo minimal heating processes, except for nisin and chemically similar bacteriocins. Bacteriocins are also sensitive to proteases in food and are degraded by proteolytic enzymes found in the gastrointestinal tract (Lück and Jager, 1997; Biswas et al., 1991).

The antibacterial action of bacteriocins produced by LAB is initiated by their ability to attach to specific cell envelope receptors (Hoover, 1993). Bruno and Montville (1993) stated that bacteriocins disrupt membranes and deplete the cellular proton motive force causing cell death in Gram-positive pathogenic cells. Most bacteriocins of LAB are bactericidal, not bacteriostatic (Hoover, 1993).

Hoover (1993) described the activity spectra and chemical properties of bacteriocins produced by LAB in great detail. Yang and Ray (1994b) listed a number of bacteriocins produced by LAB strains. As well, Kelly et al. (1996) generated a list of bacteriocin-producing bacterial isolates from variety food products, which inhibit certain strains of bacteria. There are four major types of bacteriocins which have been studied (Ouwehand, 1998), however, in this thesis only three proprietary bacteriocin products will be discussed in the succeeding sections.

2.3.4.2. Alta® 2341

Alta® 2341 is a natural, proprietary and multifunctional food ingredient made from cultured corn syrup solids through a specialised fermentation process. It enhances

flavour and extends the shelf-life of many types of food products and is labelled as a "non-chemical" ingredient (Rozum and Maurer, 1997; Quest International, 1998).

Rozum and Maurer (1997) found that 1% Alta® 2341 was able to extend cooked chicken breast meat shelf-life up to 5 weeks at refrigeration temperatures compared with untreated controls. Schlyter et al. (1993) detected ~2 $\log_{10}$ CFU/ml reduction of *L. monocytogenes* in turkey slurry using 1% Alta® 2341 following a 30 min incubation at 25°C compared to the control. The authors also showed that 0.25, 0.5 or 0.75% Alta® 2341 were unable to inhibit *L. monocytogenes* in turkey slurries alone compared to the control, but when all three levels of Alta® 2341 were combined with 0.5% diacetate, there was ~2 $\log_{10}$ unit decrease after 7 d at 25°C compared to the control. They concluded that Alta® 2341 can provide an additional hurdle for *Listeria* in poultry type products.

Quest International currently produces Alta® 2341 and other co-products of Alta®, each produced by different bacterial cultures grown on specific media.

With this composition, labelling aspect and its ability to inhibit some organisms, Alta® 2341 was thought to be useful in hampering the target organisms either alone or in combination with other natural inhibitors. Therefore, Alta® 2341 was tested in the preliminary studies.

2.3.4.3. Alta Mate®

Alta Mate®, like Alta® 2341, is also a proprietary natural multifunctional food ingredient (bacteriocin). Also like Alta® 2341, it enhances the flavour and extends the

shelf-life of many types of food products and inhibits Gram-positive bacteria including *Listeria* (Quest International, 1999).

Alta Mate® is fairly new to the food industry and little detailed research is available on this product in the public domain. Therefore, the present study was thought to be a great opportunity to gather more information on the natural antimicrobials' ability to inhibit meat spoilage organisms. Alta Mate® was used in both the preliminary testing and in the meat trials.

2.3.4.4. Other commercial bacteriocins

2.3.4.4.1. Per/Lac®

Per/Lac®, is a commercial pediocin-rich dairy ingredient powder generated by the fermentation of *Pediococcus acidilactici* PO2 in corn syrup solids or whey. The production of Per/Lac® is increased considerably in the presence of whey protein containing yeast extract. Neither ingredient alone is able to increase production of Per/Lac® (Liao et al., 1993). According to Liao et al. (1993) whey protein usually contains 4.9% lactose, 0.03% protein, 0.1% non-protein nitrogen, 0.5% ash, <0.01% fat and 0.15% lactic acid. In addition, whey protein contains some trace elements such as iron, copper, zinc and manganese, and vitamins (including vitamin A, thiamin, pyridoxin, riboflavin, calcium pantothenate, biotin, cobalamin and vitamin C). Lactose can be used as a carbon source by pediococci, and along with trace elements and vitamins in whey protein plus yeast extract, this mixture is able to support the growth and production of pediocin by *P. acidilactici* PO2 (Liao et al., 1993).

Per/Lac® has shown to be bacteriostatic because of its ability to inhibit *Listeria monocytogenes* Scott A and Ohio in heat-treated milk (Liao et al., 1993). However, Liao et al. (1993) found Per/Lac® was unable to inhibit *L. monocytogenes* in liquid whole egg, and hypothesised that Per/Lac® contained a substance that interacted with the egg and thereby eliminated the bactericidal action of Per/Lac®. They also found that increased concentrations of Per/Lac® caused corresponding increases in antilisterial activity in the treated liquid egg. Liao et al. (1993) proposed that Per/Lac® can be useful in controlling *L. monocytogenes* in selected food systems only. Rozum and Maurer (1997) have also come to the same conclusion because they found that Per/Lac® was unable to inhibit the microflora on cooked chicken breasts. The aerobic plate count values from the treated cooked chicken breasts were the same as the control. They concluded that Per/Lac® might not be as effective at controlling a broader spectrum of microorganisms.

2.3.4.4.2. Nisin

Nisin is the most common, successful and well-studied bacteriocin produced by LAB to date (Hurst and Hoover, 1993). Nisin is a polypeptide-type antibiotic derived from strains of *Streptococcus*. It consists of four similarly structured individual components composed of 29 to 34 amino acids, eight of which are sulfur-containing amino acids. There are two sub-types of nisin, namely nisin A and nisin Z; both distinguished by the amino acid in position 27: aspartic acid in nisin A and histidine in nisin Z (Lück and Jager, 1997). While nisin is produced naturally by *Streptococcus lactis*, it can also be genetically engineered (Lück and Jager, 1997). It is a generally recognised as safe (GRAS) antimicrobial and has been recently accepted for use in some

foods in the United States, for example, cheese spreads (Lück and Jager, 1997; Cassens, 1994e; Bruno, 1992). In solution, nisin stability improves with a decrease in pH value. At pH 2, nisin withstands a temperature of 121°C for 30 min without losing its activity. With pH values above 4, nisin decomposes in solutions, especially if heated. However, it has been stated that the antimicrobial action of nisin is very powerful at pH 6.5 to 6.8 even though its stability at this pH range is poor (Lück and Jager, 1997).

Nisin inhibits Gram-positive organisms, many lactic acid bacteria, streptococci, bacilli, clostridia and other anaerobic spore-forming microorganisms (Hurst and Hoover, 1993; Jay, 1992), but it has no effect against yeasts or moulds (Cassens, 1994e; Jay ,1992). Nisin allows the cytoplasmic membranes of some bacterial species to be permeable to ions and low-molecular-weight cellular compounds (Bruno, 1992). Nisin is inhibitory because it depletes the proton-motive force and exhausts the cellular ATP reserves of the cell. Nisin can also behave as a surface-active cationic detergent because it was concluded that anionic soaps cause its neutralisation (Hurst and Hoover, 1993).

The first food use of nisin was in Swiss cheese to prevent spoilage caused by *Clostridium butyricum*. Since then, nisin has been used in low-acid canned foods to inactivate endospores of both *C. botulinum* and spoilage organisms by reducing the required heat process (F_o) from ~6 down to 3 (Hurst and Hoover, 1993). This was possible because nisin is heat stable at high temperatures (121°C) for 30 min. The benefit of using nisin includes increased product quality in low-acid canned foods, even though endospores of spoilage organisms are not destroyed. Nisin prevents spore germination by acting early in the endospore germination cycle (Hurst and Hoover, 1993).

For more complete information, the reader is referred to Hurst and Hoover (1993) and Fowler and Gasson (1991) who have written very complete and detailed information about nisin.

2.3.4.5. Some other bacteria strains

The most commonly studied bacteriocins are those produced by LAB (Okereke and Montville, 1991), which includes nisin. Bacteriocins inhibit only bacteria closely related to those from which they have been formed; bacteriocins from Gram-positive bacteria are not effective against Gram-negative bacteria (Lück and Jager, 1997; Hoover, 1993). Examples include lactacins B and F produced by *Lb. acidophilus*. Lactacin B produced by *Lb. acidophilus* N2 is active against *Lb. bulgaricus*, *Lb. leichmannii*, *Lb. helveticus*, and *Lb. lactis*. As well, lactacin B is bactericidal, sensitive to proteases, and heat stable (100°C for 1h) (Hoover, 1993). *Lb. acidophilus* 88 produces Lactacin F and inhibits *Lb. fermentum* 1750 (Hoover, 1993). Like lactacin B, lactacin F is also heat stable (121°C for 15 min). Another example is helveticin J, produced by strains of *Lb. helveticus*. Helveticin J inhibits some strains of *Lb. helveticus*, *Lb. bulgaricus* and *Lb. lactis* (Joerger and Klaenhammer, 1986), and is not heat sensitive (100°C for 30 min) (Hoover, 1993).

2.3.5. Phenolics

2.3.5.1. Definition

Phenolic compounds are widespread in nature and many are found in a variety of foods such as meat and poultry products, vegetables, dairy products, alcoholic and

nonalcoholic beverages and nuts. (Maga, 1978; Davidson, 1993). Since phenolic compounds are derived from phenol, it is not surprising that these compounds exert antimicrobial activity.

Phenolic compounds found in nature vary from those with a single phenolic ring to complex polyphenolic compounds. Simple phenolic compounds include isomers of alkyl, hydroxy and methoxy derivatives of phenol. Although present in foods, the only practical use of simple phenols for preservation is found in wood smoke. Polyphenolic compounds are present in the rinds and barks of many plants. The first polyphenolic to be recognized for its fungistatic properties was tannic acid in the 1960s. Since then tannins have been tested for their antimicrobial effectiveness in some products (Davidson, 1993).

There are many research studies on the inhibitory effects of phenolic compounds described by Davidson (1993).

2.3.5.2. Liquid smoke

Liquid smoke is used as a flavouring and aroma agent, as well as a preservative in foods. It is an antibacterial agent and this was first reported in the early 1970s (Rozum and Maurer, 1997). It is thought that the phenolic components found in liquid smoke are able to inhibit bacterial growth. Faith et al. (1992) found that isoeugenol was an important phenolic in smoke that provided much of its antibacterial activity. Other researchers reported that liquid smoke components such as formaldehyde, acetic acid, creosote and high-boiling phenols exhibit bactericidal properties (Messina et al., 1988).

For this thesis work, Aro-Smoke P-50 was used. It has been used in a number a research studies with some success. Therefore, it was included in the present work with the prospect it would be able to inhibit the target organisms tested either alone or in combination with other naturally occurring antimicrobial agents. It is an aqueous extract of natural smoke flavour formulated with polysorbate 80; a mixture of oleate esters of sorbitol and sorbitol anhydrides condensed with approximately 20 moles of ethylene oxide (Anon, 1998). Aro-Smoke P-50 is water-soluble, contains hickory smoke flavour and is approved for use by Agriculture Canada (Anon, 1995; Anon, 1998). It provides good control of coagulase-positive staphylococci and is widely used on cooked and uncooked meat products to control undesirable bacteria and fungi. Aro-Smoke P-50 also is as effective as a natural antioxidant and its activity compares favourable with commercial synthetic antioxidants (Wendorff, 1981). Messina et al. (1988) found that Aro-Smoke was effective in controlling microbial growth when used in culture media. However, the level of its effectiveness in meat is lower because concentrations recommended for use on meat results in lower final concentrations of isoeugenol being transferred to the product (Rozum and Maurer, 1997).

2.3.5.3. Eugenol

Eugenol, 2-methoxy-4-(2-propenyl)phenol, is a phenolic antioxidant that has been reported by many researchers as one of the most active antimicrobials among the essential oils (Moleyar and Narasimham, 1992; Jay and Rivers, 1984; Beuchat and Golden, 1989). The hydroxyl group in eugenol is thought to be important in determining its antimicrobial activity (Shelef, 1983). Figure 1 shows the structure of eugenol.

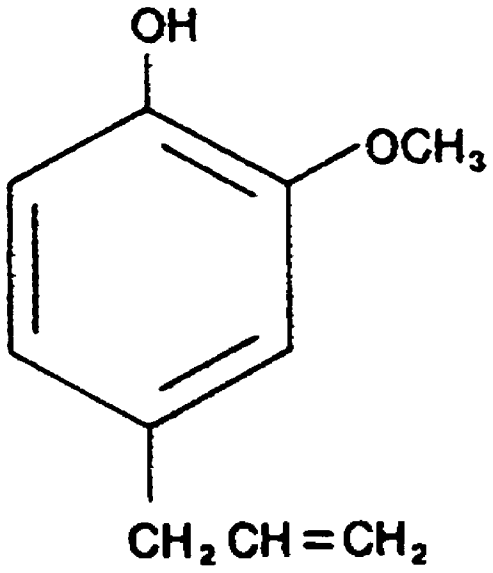


Figure 1. Structure of eugenol (Beuchat and Golden, 1989).

Eugenol is widely used as a flavouring agent in foods. It has potential for use as a preservative because it occurs in high concentrations naturally in cloves (Suresh et al., 1992; Blaszyk and Holley, 1997). It is considered an antiseptic and possesses some local anaesthetic action; for example, as a remedy for toothaches (Suresh et al., 1992).

Suresh et al. (1992) found that eugenol was able to inhibit some Gram-negative as well as some Gram-positive organisms namely: *Micrococcus* and *Bacillus spp.* Blaszyk and Holley (1998) also found that eugenol inhibited Gram-positive microorganisms. In their study, they found that *E. coli* O157:H7 and *B. thermosphacta* were inhibited by eugenol alone. They also discovered that lactobacilli were more resistant to the action of eugenol than other organisms tested such as *Lc. mesenteroides.* Eugenol was used in this work because of its natural antimicrobial characteristics and it was applied in both preliminary studies and meat trials.

2.3.5.4. Other commonly used phenolic antioxidants

Three commonly used phenolic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butylhydroquinone (TBHQ). These phenolic antioxidants are used to prevent rancidity in lipids and lipid-containing products. They also have antimicrobial activities and are effective against Gram-positive and Gram-negative bacteria; the Gram-positive being more sensitive (Branen et al., 1980). The antimicrobial activity of these phenolic antioxidants appears to depend on the presence of a hydroxyl group on the molecule, the lipid solubility of the compound and the degree of steric hindrance induced. Their mechanism of inhibition has been found to

involve alterations in: the function and composition of the cellular membrane; the synthesis of DNA, RNA, protein and lipid, and the function of the mitochondrion.

There have been many studies done on BHA, BHT and TBHQ over the years. Branen et al. (1980), Raccach (1984), Kabara (1991) and Davidson (1993) described in more detail the research done with these antioxidants.

2.3.6. Other antimicrobial agents

2.3.6.1. Potassium lactate and sodium lactate

Potassium lactate ($CH_3CHOHCOOK$) and sodium lactate ($CH_3CHOHCOONa$) are used as emulsifiers, flavour enhancers, humectants, and for pH control in a variety of foods (Brewer et al., 1991; Shelef, 1994; Nnanna et al., 1994). They are GRAS in the U.S. and permitted in meats in Canada. They can be used to extend the shelf-life of processed meats; however, potassium lactate is less preferred than sodium lactate because of the slightly bitter taste of the former (Weaver and Shelef, 1993).

There are only a few reports indicating the antimicrobial effectiveness of potassium lactate. In two separate studies, Bradford et al. (1993a and 1993b) found that between 2% and 3% potassium lactate effectively reduced populations of psychrotrophs and coliforms in fresh pork sausages. However, they did discover that microbial numbers were not reduced by the addition of potassium lactate to low-fat sausages or low-fat carrageenan-based meat patties. They suggested that the reduction of microbial numbers in fresh pork was probably due to the ability of the undissociated acid to cross cell membranes, dissociate and acidify the cell interior. Also, they suggested that potassium

lactate may reduce a_w which would result in decreased microbial growth. Shelef and Yang (1991) found that 4% potassium lactate inhibited *L. monocytogenes* in chicken and beef stored at 5°C for 15 and 20 days, respectively. Weaver and Shelef (1993) found 2 and 3% potassium lactate inhibited *L. monocytogenes* in heat sterilised pork liver sausages held at 5°C, and that the effect was listeriostatic. At an abusive temperature of 20°C, the microorganism was reduced by ~1 Log $CFU \cdot g^{-1}$ with 4% potassium lactate. They also found that potassium lactate at 2% or 3% did not affect the pH of the product, and there was only a small affect on the a_w.

There is considerable interest in using sodium lactate as a preservative for meat. Sodium lactate has been shown to have antimicrobial activity against a broad range of microorganisms. For example, Brewer et al. (1991) found that the addition of 2% or 3% sodium lactate to fresh pork sausage delayed microbial growth up to 21 and 24 days respectively. Also, 3% sodium lactate inhibited bacteria in bologna until the 8^{th} week of storage, delaying pH decline and the development of off-flavours (Brewer et al., 1992). Other researchers have also found similar results (Maca et al., 1999; Maca et al., 1997a and 1997b; Wederquist et al., 1994). Both potassium and sodium lactate were effective in inhibiting pathogens at concentrations up to 3.5% in cooked beef roasts (Stillmunkes et al., 1993; Miller and Acuff, 1994). Sodium lactate contributes to water-holding capacity and increases cooking yield as well as improving the flavour in meat products (Papadopoulos et al. (1991a, 1991b, and 1991c). The implied mechanisms of action of both lactic acid salts include feedback inhibition, intracellular acidulation, interference with proton transfer across cell membranes and altering a_w (Cassens, 1994d; Maas, 1989; de Wit and Rombouts, 1990; Shelef, 1994).

It has been found that the use of sodium or potassium lactate on meat products yielded little difference in terms of product quality and that the two salts were equal in their antimicrobial effects. The preference for the potassium salt over sodium lactate is related to the desire to limit the sodium content in meat products (Shelef and Yang, 1991).

Like some other antimicrobials used in this work, potassium lactate was chosen because of its inhibitory characteristics and success against psychrotrophic bacteria. Even though it has been shown to be inhibitory against *L. monocytogenes*, it was thought that combined with other antimicrobial agents, potassium lactate would help to reduce the growth of LAB tested in both the preliminary testing and in bologna and ham meat trials.

2.3.6.2. Phosphates

Phosphates are used quite extensively in the food industry as processing aids, for a variety of other functional purposes and recently were evaluated for their antimicrobial properties (Lück and Jager, 1997; Flores et al., 1996). They are recognized as GRAS in the U.S. and have been approved by regulatory agencies for use in meats (Shelef and Seiter, 1993). Used as an additive, phosphates: improve water binding, enhance emulsification, retard oxidative rancidity and colour deterioration, and enhance cured-colour development (Lee et al., 1994). Phosphates are therefore used to bind structured meats, to retain moisture, and to inhibit the growth of microorganims in meat, poultry and fish products (Flores et al., 1996; Lee et al., 1994). The antimicrobial action of phosphates results from their formation of complex linkages with the bivalent metals that are essential for microbial growth. This interference causes a reduction in cell division

and it reduces the structural stability of the cell wall (Lück and Jager, 1997). They have been shown to be most effective against bacteria such as *Staphylococcus aureus*, *Streptococcus faecalis*, *Bacillus subtilis* and clostridia (Tompkin, 1983; Lee et al., 1994). Phosphates also reduce the heat resistance of a number of bacteria (Hargreaves et al., 1972).

There are many types of phosphates used in the food industry. A more complete documentary of phosphates and their use is found in Shelef and Seiter (1993).

In this thesis work, disodium pyrophosphate ($Na_2H_2P_2O_7$), also known as sodium acid pyrophosphate, was used. It is approved for use in processed meats in Canada at a level of 0.5%. Major functions of disodium pyrophosphate in meat products are essentially the same as those described for phosphates generally: to stabilize meat emulsions, increase tenderness, increase hydration, retain colour and improve flavour (Shelef and Seiter, 1993). Since it is a chelator, disodium pyrophosphate was incorporated in both the preliminary tests and the meat trials to help facilitate the natural antimicrobials in hampering the target organisms.

This pyrophosphate is one of a number of polyphosphates that are negatively charged, strong polyanions that form complexes with metal ions and with positively charged proteins. It is prepared from phosphoric acid following neutralisation with sodium and is a straight-chain molecule. Figure 2 shows the structure of disodium pyrophosphate (Shelef and Seiter, 1993). According to Shelef and Seiter (1993), the antimicrobial activity of disodium pyrophosphate results from the ability of the phosphate to chelate cations that are essential for growth. Gram-positive bacteria are more sensitive to this

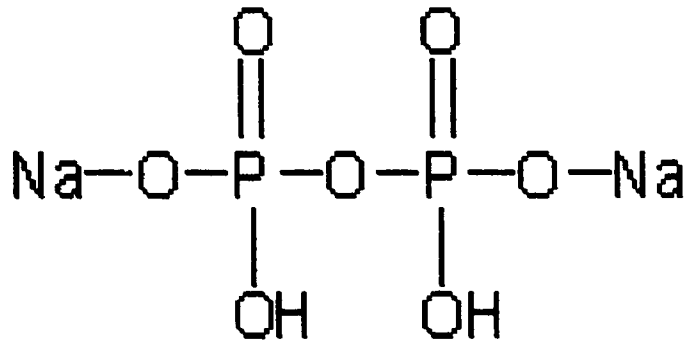


Figure 2. Structure of disodium pyrophosphate (Shelef and Seiter, 1993).

type of compound due to their higher cation requirements compared to Gram-negative bacteria.

2.3.6.3. Organic acids

Organic acids include a large group of poorly ionizable weak acids. Some are used as preservatives in food while others are used for acidification, or for taste. However, only a few are classified as food preservatives (Eklund, 1989). Microorganisms display varied tolerances to acids. For example, LAB are not only tolerant to weak lipophilic acids (lactic acid), but also produce them as a by-product of their metabolism. Some acids, for example acetic acid, may be critical to the metabolism of the lactobacilli, but inhibitory to bacilli. In instances where there is a mixed flora, the proper use of an acid in a culture medium can select for a particular group desired (Doores, 1993). Sofos and Busta (1993) stated that organic acids have a larger anti-listerial effect than their salts, due to the ability of organic acids to lower the pH. Therefore, higher concentrations of the salts from organic acids must be added to have the same effectiveness as the acids themselves (Buncic et al., 1995).

Eklund (1989), Kabara and Eklund (1991) and Doores (1993) described the mechanisms of action of different types of organic acids in detail.

There are many scientific reports describing the success of organic acids against microorganisms in foods. Ouattara et al. (1997) and Podolak et al. (1996) are just two examples where organic acids have successfully reduced or inhibited microflora in broths and meat products.

2.3.6.4. Monolaurin (Lauricidin®)

Monolaurin (Lauricidin®), is the most effective antimicrobial of the glycerol monoesters that are available (Jay, 1992). It should be noted that Lauricidin® is a proprietary form of monolaurin, with a higher level of chemical purity (94-96%) than monolaurin (45-55%). It has been primarily used in the cosmetic and pharmaceutical industry, but its approval for use in foods is currently being sought by many companies (Kabara, 1993). Lauricidin® is a monoglyceride formed from the reaction of lauric acid (C_{12}) with glycerol. It is a highly lipid soluble preservative with limited water solubility (Kabara, 1984b). Figure 3 shows the structure of monolaurin/Lauricidin®. Lauricidin® is a good emulsifier because it can be distributed between the lipophilic and hydrophilic phases of food ingredient formulations. It is a food grade fatty acid ester and chelator approved for use as a releasing agent on sausage casings. Its antimicrobial activity against most Gram-positive bacteria, fungi, yeasts, and molds has been demonstrated, but has limited antimicrobial activity against Gram-negative bacteria (Hall and Maurer, 1986). The lack of activity against Gram-negative microorganism is believed due to the different bacterial envelope possessed by Gram-negative microorganisms. The latter have a cell wall composed of two membranes, each consisting of phospholipid bilayer. The outer layer prevents monolaurin from gaining access to the inner membrane, whereas Gram-positive organisms do not possess this outer membrane. There are techniques for the removal of this outer barrier, the most effective being the addition of a chelating agent (Kabara, 1984a). Chelating agents are discussed in the next section. The antimicrobial activity of this long-chain fatty acid against Gram-positive bacteria are: a) cause lysis of protoplasts, b) cause leakage of absorbing material and protein from both bacteria and protoplasts,

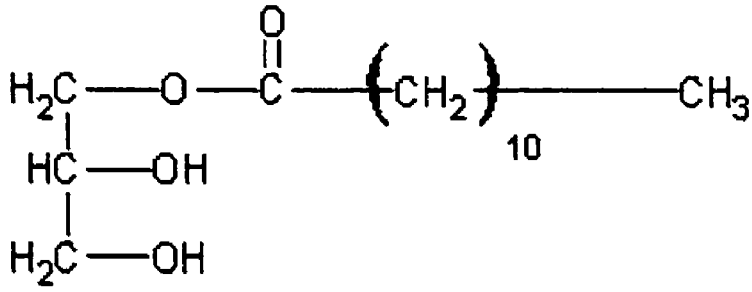


Figure 3. Structure of monolaurin/Lauricidin® (Anon, 1998).

and c) inhibit both oxygen uptake and amino acid uptake (Galbraith and Miller, 1973a). This means that the presence of monolaurin might change cell membrane permeability leading to inhibition of synthesis of macromolecules or inhibition of oxygen uptake (Galbraith and Miller, 1973b).

Monolaurin has been found to be more active at high temperatures (55°C) than at lower temperatures. In experiments designed to sanitise surfaces, Oh and Marshall (1995) found planktonic *L. monocytogenes* cells were destroyed by 50μg/ml monolaurin combined with heating at 55°C for 5 min. The authors found that temperatures below 55°C were unable to effectively inhibit the attached target organism even at the highest level of monolaurin tested (100 μg/ml). They stated that there was a 3.6 log cycle and 1.4 log cycle reduction for planktonic and adherent cells, respectively, on stainless steel chips. In another food-related study conducted by Oh and Marshall (1993) it was found that the most rapid inactivation of *L. monocytogenes* by monolaurin occurred with the highest temperature (35°C) and the lowest pH (5) combination examined. They stated that the interactions between temperature, pH, and monolaurin depend on the levels of each factor. This was backed up with a study done by the authors in 1996 again on stainless steel chips. Here, they were able to completely inactivate *L. monocytogenes* with the combination of 1% acetic acid with 50 or 100 μg/ml of monolaurin within 25 or 20 min, respectively.

There have been several studies conducted using Lauricidin®. For example, Baker et al. (1982) demonstrated that 250 ppm Lauricidin® could extend the shelf-life of mechanically deboned chicken meat, minced fish, and chicken sausage stored at 2°C for 5, 8 and 9 days, respectively. Bacteria in samples treated with Lauricidin® displayed

extended lag periods and in some cases, reduced rates of microbial growth were observed. The authors also discovered that the addition of citric acid (0.2%) with 250 ppm Lauricidin retarded the spoilage organisms and increased the shelf-life of deboned chicken meat by 7 days, minced fish by 5 days, and chicken sausage by 8 days. Researchers Wang and Johnson (1992 and 1997), Oh and Marshall (1992) and Razavi-Rohani and Griffiths (1994) concluded that 10-20, 500-1000, 10, and 96 µg/ml, respectively, monolaurin was effective against *L. monocytogenes*, however, this effectiveness depended upon the type of food and food constituents, the age of the microorganism, pH, temperature and concentration of monolaurin. Monolaurin shows promise for use as an antimicrobial in food if used in combination with other agents that might expand its spectrum of antimicrobial activity.

Lauricidin® was chosen for this work because of the extensive research done on this antimicrobial. Kabara (1993) evaluated the combination of an emulsifier-glycerol monolaurate with food grade phenolics and a metal ion chelator against a variety of organisms with success. As well, Blaszyk and Holley (1998) found success when they combined glycerol monolaurate with phenolic-based spice oil extractive (eugenol) and a chelator, EDTA. Therefore, it was thought that combining Lauricidin® with a chelator (disodium pyrophosphate) and perhaps with other natural antimicrobials, that the same success would be sought in this work.

2.3.6.5. Chelators

Chelators are used in food primarily for their antioxidative properties. They are not direct antioxidants, but rather function by eliminating pro-oxidant metals such as iron

and copper. Commonly used chelating agents are citrates, lactates, polyphosphates and ethylene diamine tetraacetic acid (EDTA) (Cassens, 1994d; Kabara, 1991).

Chelators are approved for limited use in the food industry primarily as stabilizers and sequestrants. They are not considered preservatives, but they can increase the effectiveness of other antimicrobial agents. This effect is important in overcoming the resistance of Gram-negative bacteria to chemical challenge especially during extended food storage. Early studies have shown that chelators affect Gram-negative strains by destabilising their outer membrane. Therefore, the removal of the lipopolysaccharide-containing outer phospholipid membrane from Gram-negative bacteria lowers their resistance to many antimicrobial agents (Kabara, 1991).

Many researchers have used chelating agents alone, but with minimal success in reducing microorganism growth. However, when combined with other preservatives, they become useful and are a safe addition in food preservation (Kabara, 1991; Blaszyk and Holley, 1998; Razavi-Rohani and Griffiths, 1994; Gill, 2000).

2.3.6.6. Lysozyme

Lysozyme, a water-soluble bactericidal protein, is found in many natural systems such as tears, plant tissues, milk and eggs (Cassens, 1994e; Valenta et al., 1997; Conner, 1993). Conner (1993) stated that because lysozyme has preservative properties, it could be used as a naturally occurring antimicrobial in food systems. Lysozyme is able to inhibit Gram-positive bacteria and pathogens such as *C. botulimum, L. monocytogenes* and some viruses; however, Gram-negative organisms are usually resistant (Conner, 1993; Cassens, 1994e; Valenta et al., 1997).

Lysozyme inhibitory activities against microorganisms and successful applications have been described by many authors such as: Gill (2000), Valenta et al. (1997), Conner (1993), Lück and Jager (1997) and Board and Gould (1991).

2.3.6.7. FloraCarn L-2

FloraCarn L-2 is a pure culture of *Lactobacillus alimentarius*, which has been proposed for use as a bioprotective agent. This strain does not produce hydrogen peroxide and no production of bacteriocins has been detected. According to the information from the manufacturer, FloraCarn L-2 preserves the taste and appearance of cured meat products and can grow within a wide range of temperatures, between 2°C and 40°C. It also is salt tolerant, up to 10%. As well, both raw and cooked meat products can either be sprayed with, or dipped into the commercial solution and when properly applied this results in ~10^7 cells·cm^{-2} on the surface of the product (Chr. Hansen, 1996; Björkroth and Korkeala, 1997).

FloraCarn L-2 suppresses growth of spoilage and pathogenic bacteria by competitive exclusion; for example, indigenous or natural LAB, Gram-negative microflora, *B. thermosphacta* and *L. monocytogenes* in raw, cooked, or cured meat products, which were vacuum-packaged or modified atmosphere packaged (MAP) were reduced in numbers (Chr. Hansen, 1996; Björkroth and Korkeala, 1997). However, in the study conducted by Björkroth and Korkeala (1997) it was found that when FloraCarn L-2 was incorporated into vacuum-packaged frankfurters, it did not suppress the growth of four ropy slime-producing *L. sakei* strains following inoculation.

2.3.7. Combinations of antimicrobial agents

Traditionally, it was common to use only one antimicrobial agent in a food product for the purpose of preservation, however, in recent years; the use of combined agents in a single food system has become more common. Theoretically, the use of combined antimicrobial agents provides a larger spectrum of activity, especially with increased antimicrobial action against pathogenic or spoilage organisms. It is thought that combined agents act on different species of a mixed microflora or act on different metabolic elements within similar species or strains (Parish and Davidson, 1993). This results in improved microbial control over the use of one antimicrobial agent alone. There are many types of natural inhibitory combinations used in the food industry involving antimicrobials and chelating agents. Only a selected few will be discussed here.

Gill (2000) applied a combination of 500 $mg \cdot kg^{-1}$ 1:3 lysozyme:nisin and 500 $mg \cdot kg^{-1}$ disodium ethylenediaminetetracetate (EDTA) to meat model systems. He found that the treated ham and bologna samples prevented the growth of *B. thermosphacta* up to 4 weeks and reduced the growth of *Lb. curvatus* to 3 weeks. He also found that treated bologna reduced the growth of *Lc. mesenteroides* and *L. monocytogenes* for 2 weeks and reduced the growth of *E. coli* O157:H7 for 4 weeks. On treated ham, *S.* Typhimurium growth was increased at week 3. He concluded that this combination might be used to control the growth of microorganisms found in cured meat products. He also found that cooked ham and bologna sausages coated with the combination of lysozyme, nisin and EDTA with 7% gelatin gel had a bactericidal effect on Gram-positive microorganisms up to 4 log $CFU \cdot cm^{-2}$ over a 4 week storage period.

Some researchers focused on combinations with monolaurin. Blaszyk and Holley (1998) showed that the combination of eugenol (500 and 1000 ppm), monolaurin (100-250 ppm) and sodium citrate (0.2 and 0.4%) prevented the growth of lactobacilli and pathogenic microorganisms. They discovered that this combination was unable to effectively inhibit *Lc. mesenteroides* and *L. monocytogenes* due to the presence of citrate, which may have been utilised during the course of their experiments. However, these two microorganisms were inhibited with eugenol and monolaurin only.

Other researchers studied combinations involving sodium lactate. Blom et al. (1997) found that a mixture of 2.5% (w/v) lactate and 0.25% (w/v) acetate could be used to increase the margins of safety for sliced and spreadable vacuum-packed ready-to-eat cooked meat products with similar characteristics to sliced servelat sausage and cooked ham.

A combination of a bacteriocin and organic acids studied by Scannell et al. (2000) is another example of antimicrobial combinations. In their studies, they discovered that the combinations of organic acids with lacticin 3147 (produced by *Lactococcus lactis*) enhanced the activity against *Salmonella* Kentucky and *Listeria innocua*, and was effective in the inhibition of *C. perfringens* in their meat model system. They also found that lacticin 3147 combined with either sodium citrate, or sodium lactate maintained significantly lower total aerobic bacterial numbers for the duration of the trials. They suggested that this combination could be used as an alternative to sodium metabisulfite, a preservative, which is used in fresh pork sausages in the U.K (but not allowed in North America).

CHAPTER 3

Materials and Methods

3.1. Materials

3.1.1. Bologna and Ham Sausage Samples

Maple Leaf Meats Winnipeg Ltd., kindly donated both regular bologna (5 kg) and chopped ham (5 kg) batters. The bologna consisted of: pork, mechanically separated turkey, chicken, pork or beef by-products, water, wheat flour, potato starch, salt, dextrose, spices, sodium erythorbate, sodium nitrite and smoke. The ham contained: 12.5% meat protein, pork, water, salt, sugar, dextrose, 80:20 basic sodium phosphate and hexametaphosphate, carrageenan, sodium erythorbate and sodium nitrite.

3.1.2. Antimicrobial Agents

Liquid smoke (Aro-Smoke P-50), horseradish oil, Lauricidin® (glycerol monolaurate), potassium lactate, Alta 2341® and Alta Mate® were all kindly donated. Only disodium pyrophosphate, eugenol and allyl isothiocyanate (AIT) were purchased for this work. Appendix 1 contains the origin of these antimicrobial agents.

3.1.3. Bacterial Strains

Strains No. 1, 9 and 10 were isolated from retail meats (Holley et al., 1996a) and are part of the University of Manitoba, Department of Food Science culture collection.

In this study, No. 1 was *Lc. mesenteroides* ssp. *mesenteroides*; No. 9 was *Lb. sakei*; No. 10 was *Lb. curvatus*.

3.1.4. Chemicals (Appendix 1)

3.1.5. Media and Reagents (Appendix 2)

3.1.6. Supplies and Instruments (Appendix 3)

3.2. Methods

3.2.1. Strain Maintenance

A standard procedure was followed to maintain the viability of lactic acid bacteria (LAB) cultures (Appendix 4). This procedure was used to store pure cultures at –80°C for later use.

As well, individual strains were streaked onto MRS medium, incubated anaerobically at room temperature, and then kept at 4°C. This procedure allows for continuous access to active viable LAB for up to two weeks.

3.2.2. Microorganism Identification Tests

3.2.2.1. Morphology Test

Morphological examinations of cultures were routinely performed as an indicator of culture purity. A drop of sterile distilled water was placed on a clean microscope slide. With a sterile platinum loop a colony from a culture plate was picked-off and smeared evenly over a small area of the slide to obtain well-spaced cells for examination under the microscope. A cover slip was gently placed over the smeared area and a drop of immersion oil was dispensed on top of the slip. The slide was placed on the stage of the microscope and viewed (Harrigan and McCance, 1976). Observations were carried out using a Zeiss Universal Research microscope under phase-contrast illumination.

3.2.2.2. Gram Test by KOH

Gram reactions of cells were determined using the potassium hydroxide (KOH) solubility test (Gregersen, 1978). This test distinguishes between Gram-positive and Gram-negative bacterial cells. A drop of 3% potassium hydroxide (KOH) was placed on a microscope slide. Using a sterile platinum loop, one colony was picked from a culture plate and mixed with the drop of KOH solution. After a few seconds of stirring, the loop was raised from the mixture and examined for any ropiness, or viscous thread-like slime. A positive result was regarded as ropiness or slime, which indicated the organism tested was a Gram-negative bacterium. If there was no slime, but only a watery-like suspension, the reaction yielded the conclusion that the organism was Gram-positive. This reaction is based on the solubilisation of lipopolysaccharide (LPS) from the Gram-negative cell wall which forms a viscous gel. Gram-positive cell walls do not contain enough LPS to generate a viscous solution (Gregersen, 1978).

3.2.2.3. Catalase Test

Some organisms growing on aerobic plates possess the enzyme catalase, for example *Brochothrix* spp. During the course of the meat trials, it was found that lactic acid bacteria (catalase negative) also grew on streptomycin thallous acetate actidione agar (STAA) medium, which was a selective medium for *Brochothrix* ssp. Therefore, the catalase test was used to help distinguish which type of colonies were present on STAA media.

The catalase test was performed according to Harrigan and McCance (1976). A drop of 3% hydrogen peroxide (H_2O_2) was placed on a microscope slide. Using a sterile platinum loop, a colony was picked from a culture plate and mixed with the solution. Immediate gas (O_2) production on the slide indicated a positive catalase reaction, while an absence of gas bubbles indicated a negative catalase reaction. This test was also applied to STAA media by flooding the Petri dish with 3% H_2O_2 to detect the presence of any non-LAB on the medium.

This test detects the presence of catalase, which protects bacteria from the harmful effects of hydrogen peroxide by catalytically converting H_2O_2 to water (H_2O) and O_2 (Prescott et al., 1990).

3.2.2.4. Motility Test

This test was used to determine if the unknown bacteria, discovered on VRBG plates during the course of the meat trials, were mobile. This test helps to identify the genus name of the unknowns, because not all bacteria are motile. The motility test was performed in test tubes containing the appropriate medium for the bacteria, with the exception that the amount of agar was reduced to 0.5-0.7% to allow the bacteria to move within the medium. A motility test medium can also be used. The medium within the tubes must be sterilised and cooled before the inoculation procedure. The medium was inoculated by stabbing with a needle carrying a 24 h old bacterial culture. The medium was usually stabbed horizontally from the centre of the test tube to over half the depth. The test tubes were incubated at appropriate temperatures and results were obtained generally in 24–48 h. Non-motile organisms grow only along the line of inoculation,

while motile organisms spread out from the line of inoculation and may even grow throughout the medium (BBL manual of products and laboratory procedures, 1968). Comparing tubes with an uninoculated tube (control) was helpful in evaluating the results, as was holding the test tube close to a light source.

3.2.2.5. API 20 E Test

The API 20 E test is a standardised, miniaturised version of conventional procedures for the identification of *Enterobacteriaceae* and other Gram-negative bacteria. This test was carried out in the course of meat trials when unknown bacteria were found on VRBG plates which were thought to be *Enterobacteriaceae*, since *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* did not grow on VRBG plates.

The API 20 E test was performed according to the API 20 E System product information manual (Sept. 1996). An 85% saline solution, with a pH range of 5.5-7.5, was added to a sterile test tube. With a sterile loop, a colony from a culture plate was picked and thoroughly mixed with the saline solution. Approximately 5 ml of distilled water was distributed into the incubation tray that was provided. This created a humid atmosphere during incubation. The API 20 E strip, which consisted of 20 microtubes containing dehydrated substrates, was placed onto the incubation tray. A 5 ml Pasteur pipette was used to fill the tube section of the microtubes with the bacterium suspension. This was done by tilting the incubation tray and placing the pipette tip against the side of the cupule. The manufacturer suggested to: (1) underfill the tubes labelled for ADH, LDC, ODC, H_2S and URE reactions because interpretation was best seen at this level; (2) fill both the tube and cupule section of the CIT, VP and GEL tests; and (3) after

inoculation, completely fill the cupule section of the ADH, LDC, ODC, H_2S and URE tests with mineral oil. After all microtubes were filled appropriately, a plastic lid was placed on the tray and incubated for 18-24 h at 35-37°C in an aerobic incubator. Between 18 and 24 h of incubation, all the reactions not requiring the addition of reagents were recorded on the result sheet provided. Interpretation of the reactions can be found in the Summary of Results table that is included in the API 20 E system package.

3.2.3. Strain Isolation and Incubation Conditions

All media plates were inverted during incubation. Table 1 summarises the conditions used.

3.2.4. Preliminary Tests *(in vitro)*

3.2.4.1. Preparations of Antimicrobials

Antimicrobials prepared as described below were used throughout the preliminary tests.

Lauricidin® was dissolved in 95% propylene glycol, as suggested by Hall and Maurer (1986) and Baker et al. (1982), and heated to ~70°C (Hall and Maurer, 1986). Lauricidin® was prepared at a concentration of 50,000 ppm by heating 1.5 g of Lauricidin® in 5 ml of 95% propylene glycol until Lauricidin® was completed dissolved, and then 25 ml MRS broth was used to dilute the solution to give a final volume of 30 ml. The MRS broth was used in most cases for all antimicrobials, instead of distilled water, to serve as additional nutrients for the target microorganisms.

Table 1. Strains, media and incubation conditions used during microbial analysis of microorganisms throughout *in vitro* and *in vivo* testing.

Microflora	Growth Medium	Time (h)	Temperature (°C)	Atmosphere
Total plate count	Standard plate count (SPC) agar	48	25	Anaerobic[a]
Lactic acid bacteria (LAB)	De Man-Rogosa-Sharpe (MRS) agar	48[b]	25	Anaerobic[a]
	All purpose tween (APT) agar	48	25	Anaerobic[a]
Brochothrix thermosphacta	Streptomycin thallous acetate actidione (STAA) agar	48	25	Aerobic
Heterofermentative and homofermentative LAB	M5 agar	48	25	Anaerobic[a]
Enterobacteriaceae	Violet red bile glucose (VRBG) agar[c]	24	37	Aerobic

[a] 10% CO_2 was used for anaerobic environment.
[b] sometimes a positive result was seen within 24 h.
[c] 1% (w/v) glucose was added to VRB and no overlay was applied.

Like Lauricidin®, eugenol was also prepared using 95% propylene glycol. For a stock concentration of 10,000 ppm, 0.10 ml of eugenol was dissolved in 2 ml of 95% propylene glycol and diluted with 7.90 ml of MRS broth to give a final volume of 10 ml. When eugenol was used in the spiral gradient endpoint (SGE) antimicrobial susceptibility test, a stock concentration of 182,000 ppm was prepared to give a target range of 500-1,000 ppm, which was determined by the SGE software program. Therefore, 1.82 ml of eugenol was dissolved in 3 ml of 95% propylene glycol followed by the addition of 5.18 ml of MRS broth giving a final volume of 10 ml.

Disodium ethylenediaminetetracetate was dissolved in MRS broth. For example, 1 g EDTA per 100 ml of MRS broth gave ~10,000 ppm stock concentration. Once EDTA was dissolved in MRS broth, it was standardised to pH 6.

Disodium pyrophosphate (4 g) was prepared in 50 ml MRS broth to give a final stock concentration of 80,000 ppm. Like EDTA, the pH of disodium pyrophosphate was adjusted to 6.

Sodium phosphate tripoly (10 g) was dissolved in 100 ml of MRS broth to give a final stock concentration of 100,000 ppm. It too was standardised to pH of 6.

Liquid smoke (Arrow Smoke P-50) was assumed to be at a stock concentration of 100%. Therefore it was diluted to the appropriate concentration using MRS broth. For example, 1 ml of liquid smoke was diluted with 10 ml of MRS broth to give a concentration of 100,000 ppm.

Allyl isothiocyanate was at a 95% stock concentration when purchased. It was dissolved in 0.50 g of whey protein. For example, a concentration of 2,000 ppm was

prepared by adding 0.2 ml of AIT to 0.50 g of whey protein followed by 99.8 ml of MRS broth to give a final volume of 100 ml.

Polyphosphoric acid was not diluted when used in the preliminary tests. It was assumed to be 100% concentrated. Therefore, for a stock concentration of 80,000 ppm, 8 ml of polyphosphoric acid was mixed with 92 ml of MRS broth. When polyphosphoric acid was used in the preliminary tests, it was not standardised to pH 6.

Alta® 2341 and Alta Mate® were both prepared in the same manner. For a 50% stock concentration, 12.5 g of either Alta® 2341 or Alta Mate® was mixed with 25 ml of MRS broth. It was then slightly heated (~ 40°C) to ensure complete solution.

Phosphate glass and phytic acid were also prepared in the same manner. For example, 1 g of either antimicrobial was mixed with 10 ml of MRS broth to yield a stock concentration of 100,000 ppm.

Potassium lactate was purchased as a stock concentration of 85%. Therefore, it was diluted with MRS broth to the desired concentration. For example, 2.47 ml of 85% potassium lactate dissolved in 70 ml of MRS broth gave a stock concentration of 3% or 30,000 ppm.

Morpholinoethane sulphonic acid was used in a 1% stock concentration. Therefore, 1 g of MES was dissolved in 100 ml of MRS broth.

3.2.4.2. Individual Inhibitory Factor Study

Inhibitory factor studies were conducted using broth dilution tests for determination of minimum inhibitory concentration (MIC) of test reagents. Antimicrobials alone or in combination were tested for their inhibitory effects on target

organisms. The tests were prepared according to Oh and Marshall (1992), Buncic et al. (1995) and Barry (1986).

Two different sets of tests were conducted. The first set of experiments involved three microorganisms, *Lc. mesenteroides* ssp. *mesenteroides*, *Lb. sakei* and *Lb. curvatus*, which were grown anaerobically in flasks containing 50 ml of MRS broth for 48 h at 25°C. Stock concentrations of eugenol, liquid smoke, horseradish oil, Lauricidin® and polyphosphoric acid were prepared in 250 ml flasks. JMP IN Statistical Discovery Software was used to pre-plan antimicrobial combinations. Screw capped test tubes containing specific volumes of MRS broth were sterilised before the addition of antimicrobials. The test tubes were then inoculated with the specific microorganism at a concentration of 10^3 CFU·ml^{-1} in a final volume of 10 ml each. All test tubes were vortexed-mixed prior to incubation at an abusive refrigerated temperature of approximately 8°C. Samples were removed from the test tubes and spiral-plated onto pre-poured MRS medium using an Autoplater® 4000. The Petri dishes were incubated anaerobically with mixed gas of 30:70 CO_2 and N_2, respectively, for 48 h at 25°C. Growth on the Petri dishes was examined using the Computer Assisted Spiral Bio-assay (CASBA® 4) scanner bed and image analysis software program. The time periods used were: 0, 48 h at 25°C for one set of experiments; 0, 6, 12, 24, 48 and 72 h at 8°C for another set of experiments; and 0, 12, 24, 48, 168, 288 and 360 h at 8°C for a third set of experiments. For each time period, only one test tube was used and the sample was plated in triplicate. Uninoculated and inoculated test tubes containing only MRS broth served as negative and positive controls, respectively. At each time period, these controls were also plated in triplicate and incubated as described above. Tables 2-7 indicate the

concentration of each antimicrobial added to test tubes for the various experiments conducted.

The second series of tests involved only two microorganisms, *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei*, which were grown anaerobically in flasks containing 50 ml MRS broth for two-24 h periods. The microorganism *Lb. curvatus* was eliminated from this series of tests, because it was found that *Lb. sakei* was the more persistent organism of the two. A stock concentration of each antimicrobial agent was prepared. They were Lauricidin®, disodium pyrophosphate, potassium lactate and Alta® 2341. Screw capped test tubes containing MRS broth supplemented with 2.5% (w/v) NaCl and 100 ppm (w/v) $NaNO_2$ were sterilised before the antimicrobials were added. The addition of these two salts was to mimic a standard cured meat formulation. The test tubes were then inoculated with either microorganism at approximately 10^3 colony forming units (CFU)·ml^{-1}, vortex-mixed to ensure complete mixing of all additions and stored at 8°C. At each time period, samples were taken and plated in triplicate. The plates were incubated anaerobically using CO_2 (Gas-Pak and anaerobic chamber) at 25°C for 48 h. The results were observed using the CASBA® 4 scanner bed and image analysis software program. The time period for sampling was 0, 2, 4, 6, 8, 10, 12 and 14 days. After each sampling period, the pH was measured and recorded. Uninoculated and inoculated test tubes without the additions of antimicrobials served as negative and positive controls respectively, and were also incubated and sampled during the same time periods. Table 8 outlines the antimicrobial concentrations used during this set of experiments.

Table 2. Eugenol and liquid smoke (Aro-smoke P-50) concentrations used with *Lc. mesenteroides* ssp. *mesenteroides*, *Lb. sakei* and *Lb. curvatus* at 25°C for 48 h.

Treatment Number	Eugenol (ppm)	Liquid Smoke (ppm)
1[a]	0	0
2[b]	0	0
3	700	0
4	800	0
5	900	0
6	1000	0
7	0	700
8	0	800
9	0	900
10	0	1000

[a] negative control, uninoculated MRS broth only.

[b] positive control, MRS broth inoculated with one of the target microorganisms.

Table 3. Eugenol, liquid smoke (Aro-smoke P-50) and horseradish oil concentrations used with *Lc. mesenteroides* ssp. *mesenteroides*, *Lb. sakei* and *Lb. curvatus* at 8°C for 72 h.

Treatment Number	Eugenol (ppm)	Liquid Smoke (ppm)	Horseradish Oil (ppm)
1[a]	0	0	0
2[b]	0	0	0
3	1000	0	0
4	0	1000	0
5	0	0	120

[a] negative control, uninoculated MRS broth only.

[b] positive control, MRS broth inoculated with one of the target microorganisms.

Table 4. Concentrations of eugenol and horseradish oil alone, or in combination used with *Lc. mesenteroides* ssp. *mesenteroides* at 8°C for 360 h.

Treatment Number	Eugenol (ppm)	Horseradish Oil (ppm)
1[a]	0	0
2[b]	0	0
3	0	100
4	0	60
5	0	80
6	1000	0
7	1000	100
8	1000	60
9	1000	80
10	500	100
11	500	60
12	500	80
13	750	100
14	750	60
15	750	80

[a] negative control, uninoculated MRS broth only.

[b] positive control, MRS broth inoculated with *Lc. mesenteroides* ssp. *mesenteroides*.

Table 5. Concentrations of liquid smoke (Aro-smoke P-50) and horseradish oil used with *Lc. mesenteroides* ssp. *mesenteroides* at 8°C for 360 h.

Treatment Number	Liquid Smoke (ppm)	Horseradish Oil (ppm)
1[a]	0	0
2[b]	0	0
3	0	60
4	0	80
5	0	100
6	500	60
7	500	80
8	500	100
9	750	60
10	750	80
11	750	100
12	1000	0
13	1000	60
14	1000	80
15	1000	100

[a] negative control, uninoculated MRS broth only.

[b] positive control, MRS broth inoculated with *Lc. mesenteroides* ssp. *mesenteroides*.

Table 6. Concentrations of eugenol, horseradish oil, Lauricidin^R and polyphosphoric acid used with *Lc. mesenteroides* ssp. *mesenteroides* at 8°C for 360 h.

Treatment Number	Eugenol (ppm)	Horseradish Oil (ppm)	Lauricidin^R (ppm)	Polyphosphoric Acid (ppm)
1[a]	0	0	0	0
2[b]	0	0	0	0
3	0	60	250	4000
4	0	80	250	4000
5	0	100	250	4000
6	500	60	250	4000
7	500	80	250	4000
8	500	100	250	4000
9	750	60	250	4000
10	750	80	250	4000
11	750	100	250	4000
12	1000	0	250	4000
13	1000	60	250	4000
14	1000	80	250	4000
15	1000	100	250	4000

[a] negative control, uninoculated MRS broth only.

[b] positive control, MRS broth inoculated with *Lc. mesenteroides* ssp. *mesenteroides*.

Table 7. Concentrations of liquid smoke (Aro-smoke P-50), horseradish oil, Lauricidin^R and polyphosphoric acid used with *Lc. mesenteroides* ssp. *mesenteroides* at 8°C for 360 h.

Treatment Number	Liquid Smoke (ppm)	Horseradish Oil (ppm)	Lauricidin^R (ppm)	Polyphosphoric Acid (ppm)
1[a]	0	0	0	0
2[b]	0	0	0	0
3	0	60	250	4000
4	0	80	250	4000
5	0	100	250	4000
6	500	60	250	4000
7	500	80	250	4000
8	500	100	250	4000
9	750	60	250	4000
10	750	80	250	4000
11	750	100	250	4000
12	1000	0	250	4000
13	1000	60	250	4000
14	1000	80	250	4000
15	1000	100	250	4000

[a] negative control, uninoculated MRS broth only.

[b] positive control, MRS broth inoculated with *Lc. mesenteroides* ssp. *mesenteroides*.

Table 8. Concentrations of Lauricidin^R, disodium pyrophosphate, potassium lactate and Alta^R 2341 used alone, or in combination with *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* at 8°C for 14 days.

Treatment Number	Lauricidin^R (ppm)	Disodium pyrophosphate (ppm)	Potassium lactate (ppm)	Alta^R 2341 (ppm)
1[a]	0	0	0	0
2[b]	0	0	0	0
3	250	0	0	0
4	250	4000	0	0
5	500	0	0	0
6	500	4000	0	0
7	0	4000	0	0
8	0	0	30000	0
9	0	0	0	5000

[a] negative control, uninoculated MRS + 2.5% NaCl + 100 ppm $NaNO_2$ only.

[b] positive control, MRS + 2.5% NaCl + 100 ppm $NaNO_2$ inoculated with one of the target microorganisms.

During the course of these tests and the tests below, the Autoplater® 4000 had to be reconditioned because of difficulty in proper operation. Problems occurred with the vacuum source becoming contaminated with moisture. The vacuum source was equipped, outside, with an additional Vacu-guard filter and a small flask filled with desiccant stones sealed with a two-hole rubber stopper. Two glass tubes were incorporated into the rubber stopper with one completely submerged into the desiccant stones. Both the Vacu-guard filter and the flask containing desiccant stones helped to prevent any moisture from entering into the vacuum source. Occasionally the filter and the desiccant stones were replaced.

3.2.4.3. Spiral Gradient Endpoint (SGE) Antimicrobial Susceptibility Test

The Spiral Gradient Endpoint (SGE) Antimicrobial Susceptibility test was performed according to the SGE manual from Spiral Biotech, Inc., (1993) and Razavi-Rohani and Griffiths (1994). This test is an alternative to broth and agar dilution, or spot diffusion tests that are used to evaluate the inhibitory effect of antimicrobial agents on the growth of bacterial populations. The advantage is that it allows for the testing of many different strains of bacteria against antimicrobial agent(s) using one Petri dish. It allows for greater sensitivity in quantitative determination of a minimum inhibitory concentration (MIC) endpoint and allows for establishing qualitative differences between biocidal and biostatic interactions (Schalkowsky, 1986).

The SGE test can also measure a wide range of antimicrobial concentration(s) within the gradients created on one MRS medium plate. For this work, each 15 cm Petri dish contained 70 ml of MRS agar supplemented with 2.5% NaCl and 100 ppm of

$NaNO_2$, or a combination of MRS, NaCl, $NaNO_2$ and other antimicrobial agent(s), that were pre-poured and the agar surface dried before use. The total drying time used was 2 h at room temperature; the Petri dishes were first dried for 15 min, and then inverted and drying was continued for another 1 h and 45 min. This allowed the entire agar surface to be free of any liquid or moisture. Also, this prevented mixing of added agents in residual surface moisture which could later alter the formulated gradient of agents in the agar. An important practice was to have uniform and level surfaces in the Petri dishes to attain proper dispensing of the gradients. If the medium does not have a level surface the gradients tend to become more oblong instead of circular and yield inaccurate results. Morpholinoethane sulphonic acid (MES) was also added as a buffering agent when disodium pyrophosphate was incorporated into the agar. The addition of MES helped to maintain the pH at 6, which mimics cured meat formulations.

Stock solutions were determined by entering the molecular weight and the desired inhibitor range (in ppm) of each antimicrobial agent into the computer software system. Table 9 outlines the concentration range of each antimicrobial used. Each stock solution was prepared and filter sterilised (using a syringe and membrane filter) before being spiral-plated onto media using the Autoplater® 4000. This instrument produced concentration gradients that decreased as the stylus moved from the centre to the periphery of the Petri dish, dispensing an accurate volume of stock solution. In the case of eugenol, the antimicrobial solution was vortexed before each use to avoid separation within the test tube caused by the insolubility of the essential oil in the diluent solution.

Table 9. Interaction of several antimicrobials to cause reduction in the minimum inhibitory concentration (MIC) of eugenol in spiral gradient endpoint tests with *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* at 25°C for 2 days or 8°C for 5 days.

Treatment Number	EDTA[a] (ppm)	Alta[R] 2341 (ppm)	Alta Mate[R] (ppm)	Phosphate glass (ppm)	Disodium pyrophosphate (ppm)	Potassium lactate (ppm)	Lauricidin[R] (ppm)	Eugenol (ppm)
1[b]	0	0	0	0	0	0	0	0
2[c]	0	0	0	0	0	0	0	0
3	0	5000	0	0	4000	30000	0	500-1000
4	0	5000	0	0	0	0	0	500-1000
5	0	0	0	0	0	0	0	500-1000
6	500	5000	0	0	0	30000	0	500-1000
7	0	0	5000	0	4000	30000	0	500-1000
8	0	0	5000	0	4000	0	0	500-1000
9	0	0	5000	0	0	0	0	500-1000
10	0	0	0	0	4000	0	0	500-1000
11	0	0	0	0	0	0	50-1000	0
12	0	0	0	0	0	30000	0	500-1000
13	0	0	0	4000	0	0	0	500-1000

[a] disodium ethylenediaminetetracetate.

[b] negative control, uninoculated MRS agar + 2.5% NaCl + 100 ppm $NaNO_2$.

[c] positive control, MRS agar + 2.5% NaCl + 100 ppm $NaNO_2$ inoculated with one of the target microorganisms.

After the gradients were applied, each Petri dish was marked in the centre with a non-cotton end of a sterile swab and then, using a marking pen, a cross mark was made on the bottom of the Petri dish. This mark provided a reference point when aligning the Petri dish and the SGE template together. The covered Petri dishes containing the gradients were dried for 2 h to allow sufficient time for the antimicrobial(s) to diffuse through the medium.

Each Petri dish was then placed on top of the SGE template with its centre (marked previously) aligned with the middle mark found on the template. A sterile cotton swab was dipped into MRS broth containing a 24 h old target organism and squeezed very lightly against the side of the test tube to remove any excess inoculated broth. With the swab, radial streaks of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* were then deposited on the surface of the medium. The streaks started from the outer edge of the Petri dish and followed down towards the template guides to about the 18 mm mark. There are a total of fifteen streak regions that can be applied to one Petri dish. For this work, every second streak was inoculated with the target organism, and every other streak was untouched to represent negative controls. Therefore, a total of eight inoculated streaks were on one Petri dish.

The plates were covered to prevent contamination and allowed to dry for up to 15 min. Since eugenol is volatile, 80 ml of 1.5% plain (Noble) agar was used as an overlay to cover this agent, preventing it from escaping and losing its initial concentrated gradient levels. This additional layer also provided an anaerobic environment in which LAB prefer to grow. Through many attempts, it was discovered that the overlay agar should be carefully and slowly poured as close to the edge of the Petri dish as possible to prevent

the inoculated streaks from lifting off of the medium. Control Petri dishes containing no antimicrobials were streaked with the test microorganisms only (in the same manner as previously described), and were also overlaid with plain agar.

The Petri dishes were inverted and incubated at 25°C for 48 h or at 8°C for 5 days. After incubation, visible growth was observed only on a portion of the radial streaks. The SGE template was used to measure the MIC where the growth to no-growth region occurred on the plate. The SGE template is shown in Figure 4. The results were recorded and entered into the computer software where the MIC for that particular antimicrobial agent was calculated. The software used a number of parameters; for example, medium thickness (4.6 mm) and incubation period (48 h). Each experiment was performed in triplicate.

To detect the viable $CFU \cdot ml^{-1}$ present in inocula the Autoplater® 4000 was used. Cultures of each strain were spiral-plated, in triplicate, on MRS medium and incubated anaerobically at 25°C for 48 h. The Petri dishes were then counted either by hand, or by using the electronic scanner with image analysis software program. This procedure will be discussed in more detail in the microbiology analysis section of this chapter.

3.2.4.4. Agar and Antimicrobial Diffusion Tests

3.2.4.4.1. Agar Spot Test

The agar spot test was carried out according to Spelhaug and Harlander (1989), and Barry (1986). This test was used to initially determine bacterial susceptibility to antimicrobial agents.

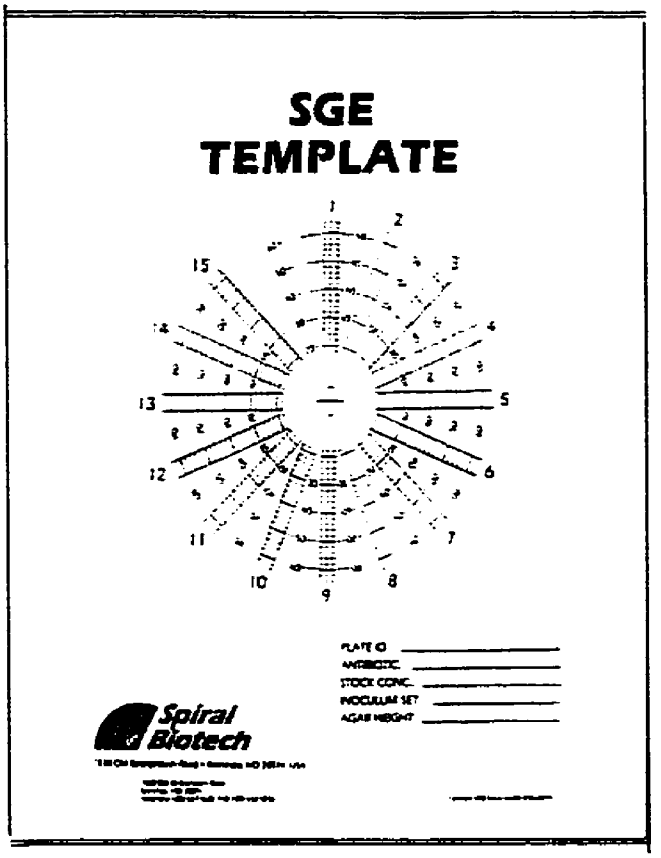


Figure 4. Spiral gradient endpoint (SGE) template used to determine the minimum inhibitory concentrations (MIC) of various antimicrobial agents against *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* (Spiral Biotech, Inc., 1993).

Each Petri dish contained 25 ml of MRS medium supplemented with 2.5% NaCl and 100 ppm $NaNO_2$, with or without the addition of an antimicrobial agent. The plates were dried for 2 h before the surface was inoculated with a 24 h old test microorganism. Surface inoculation was carried out by either spreading with a hockey stick (glass rod) or by the "lawn" method. The lawn method was performed using the Autoplater® 4000. Each inoculated Petri plate contained approximately 10^8 CFU·ml^{-1}, at volumes of 100 and 300 µl for the hockey stick method and 20 µl was used for the lawn method. Positive controls were Petri dishes inoculated the same way without antimicrobials, and negative controls were without antimicrobials or bacteria (just the test medium itself). All Petri dishes were then dried for 15 min at room temperature.

The antimicrobial agents tested (Table 10) were made up in a series of test tubes, each containing a different concentration. Lauricidin® and eugenol concentrations decreased by 100 ppm at each subsequent dilution while EDTA, disodium pyrophosphate and sodium phosphate tripoly concentrations were decreased by 1000 ppm. A blank (plain MRS broth), containing no antimicrobial agent, was also prepared for this test. The concentrated antimicrobial was thoroughly vortex-mixed before 5 µl aliquots were spotted onto the surface of the medium. Each spot was spaced approximately 2 cm apart per plate. Each experiment was carried out in triplicate. If the antimicrobial was volatile, a thin (30 ml) 1.5% agar layer was carefully poured on top to decrease the chances of the antimicrobial from being volatilised. The Petri dishes were incubated at 25°C for 24-48 h or 8°C for up to 7 days. A clear zone extending laterally from the border of the spot denoted inhibition.

Table 10. Antimicrobial concentrations used alone, or in combination during agar spot tests with *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* at 25°C for 48 h or 8°C for 7 days.

Treatment Number[a]	Lauricidin[R] (ppm)	Eugenol (ppm)	Disodium pyrophosphate (ppm)	EDTA[d] (ppm)	Sodium phosphate tripoly (ppm)
1[a]	0	0	0	0	0
2[b]	0	0	0	0	0
3	100-500[e]	0	0	0	0
4	250[c]	100-1000[e]	0	0	0
5	500[c]	100-1000[e]	0	0	0
6	0	0	1000-10000[f]	0	0
7	0	0	0	0	1000-10000[f]
8	0	0	0	1000-10000[f]	0

[a] negative control, uninoculated MRS broth + 2.5% NaCl + 100 ppm $NaNO_2$ only.

[b] positive control, MRS broth + 2.5% NaCl + 100 ppm $NaNO_2$ inoculated with one of the target microorganisms.

[c] antimicrobial incorporated into MRS medium before solidification.

[d] disodium ethylenediaminetetracetate.

[e] concentrations increased by intervals of 100 ppm.

[f] concentrations increased by intervals of 1000 ppm.

The size of the zone of inhibition was proportional to the log of the concentration of the antimicrobial.

Several alternative procedures were used during the course of these experiments. First, if a type of phosphate was tested, 1% (w/v) MES was incorporated into the MRS broth mixture as a buffering agent to maintain the pH at 6. Secondly, sometimes molten agar (0.5%) was mixed with the volatile antimicrobial within the test tubes to help prevent the agent from dissipating into the air. Lastly, Lauricidin® sometimes was incorporated into the MRS medium.

3.2.4.4.2. Well Diffusion Test

The well diffusion test was also used to determine the inhibitory effects of antimicrobials against target organisms. This was done by incorporating the volatile agents into agar and the mixtures were placed into wells. Results obtained can be similar to those of the agar spot test and in both techniques zones of inhibition were measured to determine the inhibitory effect of an antimicrobial agent.

Strains were grown anaerobically in MRS broth at 25°C for 24 h. The Petri dishes contained 25 ml of MRS medium supplemented with 2.5% NaCl and 100 ppm $NaNO_2$. The test strain, with a final concentration of approximately 10^6 CFU·ml^{-1}, was incorporated into the agar medium mixture after being cooled to 47-49°C and vortexed quickly. The medium was then poured into Petri dishes and allowed to dry for 2 h. Wells were cut from the agar by using the larger hollow end of a sterile pasteur pipette and applying a slight vacuum to remove the plug from the medium. A 15 µl amount of 0.5% plain molten agar was used to seal the bottom of the well.

All the antimicrobial solutions were filter sterilised (using a syringe and a 0.45µm membrane filter), placed in separate test tubes and vortex-mixed before each use. A 50 µl aliquot of concentrated antimicrobial solution was applied to every well, at intervals of 100 ppm. Subsequently, 45 µl of 0.5% molten agar was quickly poured on top. This was done to delay vapourisation of volatile antimicrobials from the medium. Once all the wells were charged with antimicrobials, 1.5% molten agar was used as an overlay to fill the wells and provide an additional barrier to delay vapourisation of volatile antimicrobials, and it also served to create an anaerobic environment during testing. Table 11 outlines the experimental design used in the well diffusion test.

Another well diffusion method was used to see if useful results could be obtained by modifying the previous procedure. Each Petri dish contained 25 ml of 1.5% agar (Noble) instead of MRS agar. Wells were made as indicated above which included use of 15µl of 1.5% agar (Nobel) to seal the bottom of the wells. In each well, 0-1000 ppm at intervals of 100 ppm AIT (Table 11) was added immediately following a combination of 47-49°C MRS agar supplemented with 2.5% NaCl and 100 ppm $NaNO_2$ and 24 h target bacteria (approximately 10^6 $CFU \cdot ml^{-1}$). An overlay of 1.5% agar (Noble) was then used, again to serve as an extra layer to prevent AIT from escaping into the air, and to provide an anaerobic environment.

The Petri dishes were then incubated at 25°C for 24–48 h, or up to 7 days at 8°C. Results were recorded when observations of clear inhibition zones were made around the wells (Barry, 1986; Zhang, 1998). This test was carried out in triplicate. Media without microorganisms and antimicrobials served as negative controls, while positive controls contained the target organism without the antimicrobials.

Table 11. Concentrations of allyl isothiocyanate (AIT) used in well diffusion tests with *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* at 25°C for 48 h or 8°C for 7 days.

Treatment Number	AIT (ppm)
1[a]	0
2[b]	0
3	500-1000[c]

[a] negative control, uninoculated MRS agar + 2.5% NaCl + 100 ppm $NaNO_2$ only.

[b] postive control, MRS agar + 2.5% NaCl + 100 ppm $NaNO_2$ inoculated with one of the target microorganisms.

[c] concentrations increased by 100 ppm.

3.2.4.4.3. Disc Assay Test

The disc assay test was performed according to Acar and Goldstein (1986) and Barry (1986). This test is similar to the agar spot and well diffusion tests where the zones of inhibition by antimicrobials are determined. This test was used to determine the MIC of the test antimicrobials against the target organisms.

Basically, 25 ml MRS agar medium supplemented with 2.5% NaCl and 100 ppm $NaNO_2$ was tempered to 45-47°C, inoculated with the target organism at 10^8 CFU·ml^{-1} and poured into Petri dishes. Alternatively, the inoculum was applied using a hockey stick or by the lawn method (Autoplater® 4000) using pre-poured solidified MRS agar. Antimicrobials were also incorporated directly into the medium mixture to determine the range of sensitivity to be examined in subsequent tests.

Paper antibiotic test discs (6.35 mm BBL) were placed on top of the medium surface using sterile tweezers. The discs were arranged at least 15 mm from each other and from the edge of the plate. This prevented the zones of inhibition from overlapping one another.

All combinations of antimicrobials were prepared in test tubes and vortex-mixed before each use. Concentrations of all antimicrobials prepared in test tubes increased by intervals of 100 ppm. Table 12 outlines the antimicrobials used for this test. Using a pipette, 30 µl of the concentrated antimicrobial agent was dispensed on top of the disc. For every plate, one disc containing MRS broth plus NaCl and $NaNO_2$ served as a negative control. The Petri dishes were then overlaid using 0.5% (w/v) molten agar to delay release of volatile antimicrobials from the mixture and to provide an anaerobic

Table 12. Antimicrobials used alone, or in combination to determine the minimum inhibitory concentrations in disc assay tests with *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* at 25°C for 48 h or 8°C for 7 days.

Treatment Number	Eugenol (ppm)	Disodium pyrophosphate (ppm)	Potassium lactate (ppm)	Alta[R] 2341 (ppm)	Alta Mate[R] (ppm)	Lauricidin[R] (ppm)	AIT[b] (ppm)	EDTA[c] (ppm)	Phosphate glass (ppm)	Phytic acid (ppm)
1[d]	0	0	0	0	0	0	0	0	0	0
2[e]	0	0	0	0	0	0	0	0	0	0
3	500-1000[a]	4000[f]	30000[f]	0	5000[f]	0	0	0	0	0
4	100-1000[a]	0	0	0	0	250[f]	0	4000[f]	0	0
5	100-1000[a]	0	0	0	0	500[f]	0	4000[f]	0	0
6	400-1000[a]	0	0	0	0	0	0	0	0	0
7	400-1000[a]	4000[f]	0	0	0	0	0	0	0	0
8	400-1000[a]	4000[f]	30000[f]	0	0	0	0	0	0	0
9	400-1000[a]	4000[f]	0	5000[f]	0	0	0	0	0	0
10	400-1000[a]	4000[f]	30000[f]	5000[f]	0	0	0	0	0	0
11	400-1000[a]	4000[f]	40000[f]	0	0	0	0	0	0	0
12	400-1000[a]	4000[f]	0	10000[f]	0	0	0	0	0	0
13	400-1000[a]	4000[f]	40000[f]	10000[f]	0	0	0	0	0	0
14	0	4000[f]	0	0	0	100-5500[a]	0	0	0	0
15	0	4000[f]	0	5000[f]	0	0	100-5500[a]	0	0	0
16	0	4000[f]	30000[f]	5000[f]	0	0	100-5500[a]	0	0	0

[a] concentrations were tested at 100ppm intervals.

[b] allyl isothiocyanate.

[c] disodium ethylenediaminetetracetate.

[d] negative control, uninoculated MRS agar + 2.5% NaCl + 100 ppm $NaNO_2$ only.

[e] positive control, MRS agar + 2.5% NaCl + 100 ppm $NaNO_2$ inoculated with one of the target microorganisms.

[f] antimicrobial incorporated into MRS medium mixture, otherwise they were applied to discs on the agar surface.

Table 12. continued.

Treatment Number	Eugenol (ppm)	Disodium pyrophosphate (ppm)	Potassium lactate (ppm)	Alta[R] 2341 (ppm)	Alta Mate[R] (ppm)	Lauricidin[R] (ppm)	AIT[b] (ppm)	EDTA[c] (ppm)	Phosphate glass (ppm)	Phytic acid (ppm)
17	0	4000[f]	30000[f]	0	0	0	100-5500[a]	0	0	0
18	0	0	30000[f]	0	0	0	500-1700[a]	0	0	0
19	0	0	30000[f]	500[f]	0	0	100-1700[a]	0	0	0
20	0	0	0	500[f]	0	0	500-1700[a]	0	0	0
21	0	0	0	0	1000-5000[a]	0	0	0	0	0
22	0	0	0	0	1000-5000[a]	250[f]	0	0	0	0
23	0	0	0	0	0	250[f]	0	1000-4000[a]	0	0
24	0	0	0	0	0	250[f]	0	0	0	1000-4000[a]
25	0	0	0	0	0	500[f]	300-1700[a]	0	0	0
26	0	0	0	0	0	100-5500[a]	0	0	0	0
27	0	0	0	0	0	0	500-1700[a]	0	0	0
28	0	0	0	0	0	0	0	1000-4000[a]	0	0
29	0	0	0	0	0	0	0	0	1000-4000[a]	0
30	0	0	0	0	0	0	0	0	0	1000-4000[a]

[a] concentrations were tested at 100 ppm intervals.

[b] allyl isothiocyanate.

[c] disodium ethylenediaminetetracetate.

[f] antimicrobial incorporated into MRS medium mixture, otherwise they were applied to discs on the agar surface.

environment. It should be noted that the antimicrobials with one concentration (Table 12) were incorporated into the MRS medium, and antimicrobials with concentration ranges were placed onto the paper discs. The Petri dishes were then dried for 15 min prior to incubation at 25°C for 24-48 h or incubated at 8°C for up to 7 days. The tests were carried out in triplicate and the target organisms were checked for concentration, viability and purity by plating on MRS agar using the Autoplater® 4000.

Results were recorded where the zone of inhibition or no growth was observed around the discs. Zones were measured to the nearest millimetre (mm) with a ruler from the edge of the disc to the outermost rim of the zone which exhibited a homogeneous and uniform zone of no growth. Since the disc assay involves three-dimensional diffusion, the size of the zones is dependent upon the cumulative rates of diffusion as well as inhibitor potency (Acar and Goldstein, 1986).

It was found during this work that using dark field illumination enhanced the ability to see the zones more clearly. As well, 1% MES was used when disodium pyrophosphate, EDTA, or phosphate glass were incorporated into the MRS medium.

After several attempts were made, adding a drop (5 µl) of 0.5% molten agar to the underside of the disc helped to prevent the disc from lifting and moving away when the overlay was applied.

Table 13. Antimicrobial concentrations used alone, or in combination in determining minimum inhibitory concentration (MIC) in microtitre plate assays with *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* at 25°C or 8°C for 7 days.

Treatment Number[a]	Lauricidin[R] (ppm)	Disodium pyrophosphate (ppm)	Propylene glycol (ppm)	Alta Mate[R] (ppm)	Potassium lactate (ppm)
1[a]	0	0	0	0	0
2[b]	0	0	0	0	0
3[c]	0	0	50-1000[e]	0	0
4[c]	50-1000[e]	0	0	0	0
5[c]	50-1000[e]	0	0	5000	0
6[c]	50-1000[e]	4000	0	0	0
7[c]	50-1000[e]	0	0	0	30000
8[cd]	50-1000[e]	4000	0	5000	0
9[cd]	50-1000[e]	4000	0	5000	30000

[a] negative control, uninoculated MRS broth + 2.5% NaCl + 100 ppm $NaNO_2$ only.

[b] positive control, MRS broth + 2.5% NaCl + 100 ppm $NaNO_2$ inoculated with one of the target microorganisms.

[c] these treatment numbers were prepared with and without the target microorganisms.

[d] tests were incubated at 25°C or 8°C for 4 days only.

[e] concentrations increased by 50 ppm.

3.2.4.5. Growth Inhibition Test

The growth inhibition test was adapted from Blaszyk and Holley (1998) and Ouattara et al. (1997). This test was used to determine the MIC of individual and combinations of antimicrobial agents(s) against test organisms.

The experiments were performed in 96-well (U-shaped bottom) microtitration plates. The MRS broth was supplemented with 2.5% NaCl and 100 ppm $NaNO_2$, as well as combinations of antimicrobials incorporated into the broth (Table 13). All the combinations were prepared in individual test tubes and vortex-mixed before each use. When disodium pyrophosphate was incorporated into the broth, 1% MES was added as a buffering agent to maintain the pH at 6.

Antimicrobial concentrations were prepared in separate test tubes containing MRS broth supplemented with 2.5% NaCl and 100 ppm $NaNO_2$, with concentrations increasing by 50 ppm. Aliquots of 200 µl of inoculated and uninoculated broths, with the various concentrations of antimicrobials, were distributed into the wells using a calibrated pipetter. The inoculated broths contained approximately 10^6 CFU·ml^{-1} of the target organism. Positive and negative controls consisted of MRS broth, NaCl and $NaNO_2$ with or without the bacterial strain and were dispensed at the last column of every microtitre plate. The experiments were prepared in triplicate with each concentration filling 4 wells per plate. Using a multi-dispensing pipetter, mineral oil (50 µl) was used to cover each well, to provide an anaerobic environment and to prevent volatile antimicrobials from dissipating into the air. The plates were incubated at 25°C or 8°C for up to 7 days on a rotary shaker. The plates were read at a single wavelength of 655 nm every 24 h using a microplate reader.

During the course of testing several antimicrobials, it was found that Lauricidin® tended to form white crystals at the bottom of some wells at 8°C within 24 h, indicating that it may have precipitated during the course of incubation. Therefore, a study was carried out to determine the concentration of Lauricidin® at which crystals formed. Different concentrations of Lauricidin® (100-500 ppm) were combined with MRS medium, poured into Petri dishes, allowed to solidify and then incubated at 8°C for 24 h. Crystals that were visually observed were recorded.

3.2.5. Meat Model Systems *(in vivo)*

3.2.5.1. Product Preparation

Batches of fresh, commercially prepared regular bologna and chopped ham were obtained in from Maple Leaf Meats Winnipeg, the day of manufacture and quickly stored at – 40°C to be used later in the production of sausages.

The day prior to making sausages, hog casings were rinsed in distilled water and then allowed to soak overnight at 4°C to remove the salt. Both types of meat were thawed in a 4°C cooler overnight. The next day, the batters were divided into two equal lots. One lot became the untreated batter, while the other became the treated batter.

There were two slightly different approaches in making the bologna and ham sausages, because at the beginning phosphates incorporated into the chopped ham by the manufacturer were not taken into account, as well the first meat trial failed to inhibit both target organisms beyond 5 weeks. Therefore, in the first meat trial, the inhibitor treatments contained 0.4% disodium pyrophosphate, which was added to both the

bologna and ham sausages. In the second trial no disodium pyrophosphate was added to the ham. Table 14 illustrates the experimental treatments used in both meat trials.

In the first meat trial, the untreated sausages were commercial meat batters stuffed into hog casings with or without inhibitor additions to both types of batter. In the second meat trial, 0.4% disodium pyrophosphate was added only to the untreated bologna emulsion because the ham already contained 0.25-0.35% commercially added phosphates (a mixture of 80:20 dibasic sodium phosphate and hexametaphosphate). Again, the untreated uninoculated and inoculated sausages served as negative and positive controls, respectively. Figure 5 shows how the meat trials were set up, and Table 14 describes in detail concentration of each antimicrobial used.

It was found that the commercial ham batter contained <2.5 cm whole pieces of meat; therefore, it was chopped for ~5 min into smaller pieces using a bench top food chopper. Once pieces were <0.5 cm, the antimicrobials were added slowly and mixed thoroughly within the batter for approximately 2 min. The bologna mixture, on the other hand, was mixed in a hobart mixer along with the antimicrobials for approximately 2 min because the bologna emulsion was stiffer than the ham and could not be properly mixed in the chopper. The temperature of the batters was monitored during this step to ensure that it did not increase beyond 10°C, because actinomysin is affected (Cassens, 1994). As the temperature increases, the actinomysin breaks down and eventually the emulsion stability is affected producing a "soupy" like batch of meat which does not "set" during cooking.

Table 14. Final concentrations (% w/w) of combined agents used to extend the shelf-life of cooked cured vacuum-packaged bologna and ham stored at 8°C.

Agents	Experimental Treatments			
	Trial 1			
	Bologna		Ham	
Alta Mate®	0.5	0.5	0.5	0.5
Eugenol	0.04	0.035	0.04	0.035
Potassium lactate		3		3
Disodium pyrophosphate		0.4		0.4
	Trial 2			
	Bologna		Ham	
Alta Mate®	1	1	1	1
Eugenol	1	1	1	1
Potassium lactate		3		3
Disodium pyrophosphate	0.4	0.4		

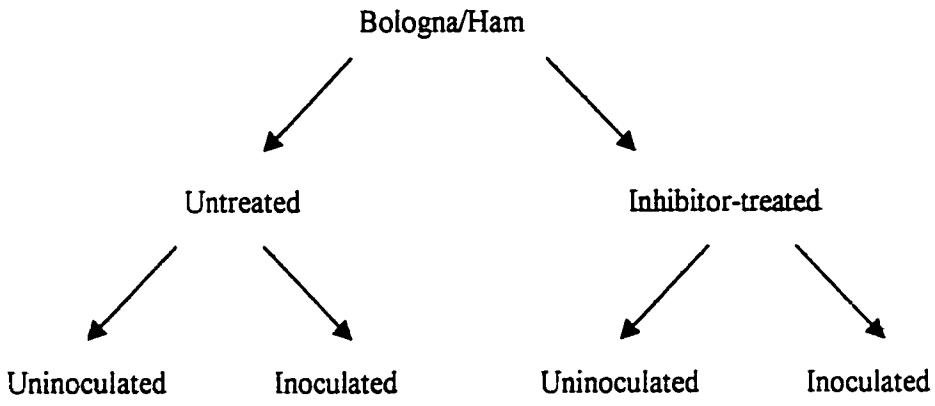


Figure 5. The standard plan of all meat trials conducted.

As shown in Table 14, there were two different combinations of inhibitor-treated bologna and ham sausages made in the meat trials. In the first meat trial, a combination that consisted of 0.5% (w/w) Alta Mate® and 400 ppm (w/w) eugenol was incorporated into both types of meat batters. The second combination consisted of 0.5% (w/w) Alta Mate®, 350 ppm (w/w) eugenol, 3% (w/w) potassium lactate and 0.4% (w/w) disodium pyrophosphate. During the second meat trial, the concentrations of inhibitors in the combinations were increased. The first combination consisted of 1% (w/w) Alta Mate® and 1000 ppm (w/w) eugenol. The second combination consisted of 1% (w/w) Alta Mate®, 1000 ppm (w/w) eugenol and 3% (w/w) potassium lactate. Disodium pyrophosphate (0.4% w/w) was only added to the bologna mixtures.

The Alta Mate® and eugenol combination was dissolved in 50 ml of distilled water. This facilitated even distribution of the antimicrobials in the meat mixture. For the second combination, Alta Mate®, eugenol and disodium pyrophosphate (in the case of bologna) were added directly to 50 ml of potassium lactate without further dilution.

After each batch of meat was prepared, it was stuffed into sausage casings using a hand-cranked sausage stuffer and clipped at approximately 15-30 cm lengths with metal clip rings. The preparation and stuffing of the batters were carried out in a 4°C environment to improve handling and prevent fat smearing onto the casings. Each sausage was then vacuum-packaged in a separate Winpak Deli #1 high-O_2 barrier bag before cooking. This step reduced the probability that eugenol, which is volatile, would escape into the air during cooking.

Once made and vacuum-packaged, the sausages underwent a three step cooking process in jacketed steam kettles. This ensured that the internal region of each sausage

was properly cooked to a temperature of 69°C without destabilising the emulsion. The temperature intervals used were those used by Maple Leaf Meats in their cooking processes (52.8°C, 63.9°C and 75°C). Each interval was timed for a period of 20 min. After cooking, the sausages were held in a 4°C cooler for 0.5 h before being stored at -20°C.

3.2.5.2. Preparation for Meat Trials

Bologna and ham sausages, both untreated and treated, were thawed overnight at 4°C and then cut into thick slices or "coins" using a sterile knife and sterile cutting board. The meat coins measured 29-31 mm in diameter, 12-14 mm in thickness with a surface area of approximately 28.7 cm^2. Each coin weighed 10 g ± 1 g. Five coins were prepared for each time interval; three coins were used for microbial analysis and two coins were used for colour measurement. Some untreated and treated coins were inoculated with *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei*. Both organisms were grown anaerobically in MRS broth at 25°C for 48 h before 0.5 ml of each organism was mixed together in 1 L of 0.1% peptone water. This ensured a combined concentration of 10^4 CFU·ml^{-1} in the peptone water and 10^3 CFU·cm^{-2} on the meat surface. After soaking for 30 s the inoculated coins were removed from the solution using sterile tweezers and allowed to dry on sterile filter paper for 15 min in a laminar airflow hood before being packaged. As mentioned before, five coins were placed separately in Winpak Deli #1 bags for each time period, vacuum-packaged (at –0.9 atm.) and stored at 8°C for later sampling. The sampling was conducted once weekly for 9 weeks, or until spoilage (approximately10^7-10^9 CFU·cm^{-2}) had occurred.

3.2.5.3. Sampling Sausage Coins

Every week, three coins were removed from 8°C storage and analysed. Each coin was aseptically removed from the vacuum-package and was massaged in a Stomacher bag along with 90 ml sterile 0.1% peptone water for a period of approximately 2 min. This produced a 10^{-1} dilution. Dilutions of 10^{-3} and 10^{-5} were then made using 9 ml sterile 0.1% peptone water. The three dilutions were plated on pre-poured media using the Autoplater® 4000 and incubated at proper temperatures and conditions. Sampling for microbiological content was performed using five different types of media. They were: M5, APT, SPC, VRBG and STAA. The strain isolation and incubation conditions noted in section 3.2.3. contains information on the incubation temperatures and conditions used for this work.

After incubation, bacterial colonies on Petri dishes were counted using the CASBA® 4 computer system. The microbiology analysis section (3.2.5.7.) describes in detail the plating and counting procedures used. The results from the microbiology analysis were converted from $CFU \cdot ml^{-1}$ to $CFU \cdot cm^{-2}$ of coin surface by dividing $CFU \cdot ml^{-1}$ by a factor of 0.31922. The latter number was obtained by dividing the average surface area of a coin (28.73 cm^2) by the volume of the first dilution (90 ml).

3.2.5.4. Visual and Odour Analysis

Every week each treatment was visually observed for: increase in drip loss, colour change and loss of vacuum. As well, each bag was opened quickly and carefully smelled for off-odours before microbial analysis was carried out. Visual observations and odour detection were noted and recorded.

3.2.5.5. Colour Determination

Colour determinations were made to detect changes that might have occurred when antimicrobials were added to bologna and ham sausages. Two coins were analysed for colour using the Miniscan spectrocolourimeter, which was standardised using a white tile (L = 92.2, a = -1.0, b = 0.1). Coins were placed between two rectangular pieces of plexi-glass plate (10.2 cm x 12.7 cm x 25.4 cm) placed on top of a white cutting board. Three hunterlab values were measured on each side of the coins. Therefore, four sets of three values were obtained per treatment every week. The three values measured were: "L", which designates the lightness of the sample where 100 represents white and zero represents black; "a", which indicates redness when positive or greenness when negative; and "b", which indicates yellowness when positive or blueness when negative (Miniscan spectrocolourimeter manual; Pomeranz and Meloan, 1994).

3.2.5.6. Determination of pH and A_w

The pH represents the acidity, or hydrogen ion exponent of an aqueous solution, in this case the meat solution (McQuarrie and Rock, 1987; Troller and Scott, 1992). The pH was used to monitor the growth of LAB due to their production of lactic acid which, at peak bacterial numbers, could decrease the pH of the meat. The pH was measured after each coin was sampled per treatment per week at the 10^{-1} dilution only. Before the pH was measured, the pH meter was standardised using 4, 7 and 10 standardised pH solutions (Troller and Scott, 1992).

Water activity analyses were performed according to Troller and Christian (1978). A Decagon a_w machine was first standardised using two solutions: distilled water

(a_w=1.0) and saturated potassium sulphate (K_2SO_4) (a_w=0.97). Both solutions were prepared a day earlier at room temperature (approximately 25°C) prior to being poured into plastic cups (4 cm x 1 cm) to standardise the machine. Samples of uninoculated sausages at room temperature were cut to fit the plastic cup diameter (4 cm) and only fill half the height (0.5 cm). The samples were then placed in the machine, and the a_w and temperature were measured and recorded.

3.2.5.7. Microbial Analysis

Microbial analyses of the sausage coins were conducted according to the spiral-plater system manual and after Manninen et al. (1991). This semi-automated method has been found to be equivalent to the standard pour plate procedure for quantifying the level of microorganisms in samples (Swanson et al., 1992).

Microbial analysis involved placing a dilution of the sample being spiral-plated (Autoplater® 4000 model) onto selective or differential agar pre-poured in Petri dishes. A Petri plate was placed on a turntable of the machine and a stylus tube pulled 250 µl of the sample from a 5 ml plastic beaker cup and dispensed 50 µl onto the surface of the medium in an Archimedes spiral pattern. During this process, the stylus was driven from the inner to outer most regions of the plate. Consequently, the amount of sample decreased as the spiral moved out towards the edge of the Petri dish. Each coin was plated in triplicate by this method.

Once the inoculated agar was dried (approximately15 min), plates were inverted and incubated at the desired temperature, time and atmosphere. See Table 1 for complete

details. Figure 6 shows what the surface of the medium would look like after it was spiral-plated and incubated for a period of time.

After incubation, Petri dishes were either counted by hand using a specially designed pie-shaped grid that relates volume to area (Figure 7), or by using an electronic scanner bed where the data was collected by the computerised image analysis system known as CASBA® 4. Counting the plate by hand using a pie-shaped grid was used for spiral plates that had well separated colonies in the outer spirals. Counting began at the outer segment (number 8 on the grid) in either quadrant A or B (see Figure 7) until at least 20 colonies were counted. It was important to continue to count to the end of the final segment. Once one segment was counted, the same segment on the opposite side of the plate (in the same letter sector as the first count) was counted. The number of counted colonies from both sides (should equal at least 40 unless counting entire plate) was divided by the volume constant found for the last segment counted (given in the Spiral Biotech manual) and multiplied by 1000 to produce a $CFU \cdot ml^{-1}$ value. For example, if for a 10^{-4} sample dilution the total counts in opposite segments 8, 9 and 10 for a 100 mm plate were 28 and 32, respectively, the $CFU \cdot ml^{-1}$ was calculated as follows: $(28+32)/5.500\mu l \times 1000 \times 10^{-4} ml = 1.09 \times 10^{3}$ $CFU \cdot ml^{-1}$.

As mentioned previously, five selective and differential media were used for the microbiological analyses (M5, APT, VRBG, STAA and SPC).

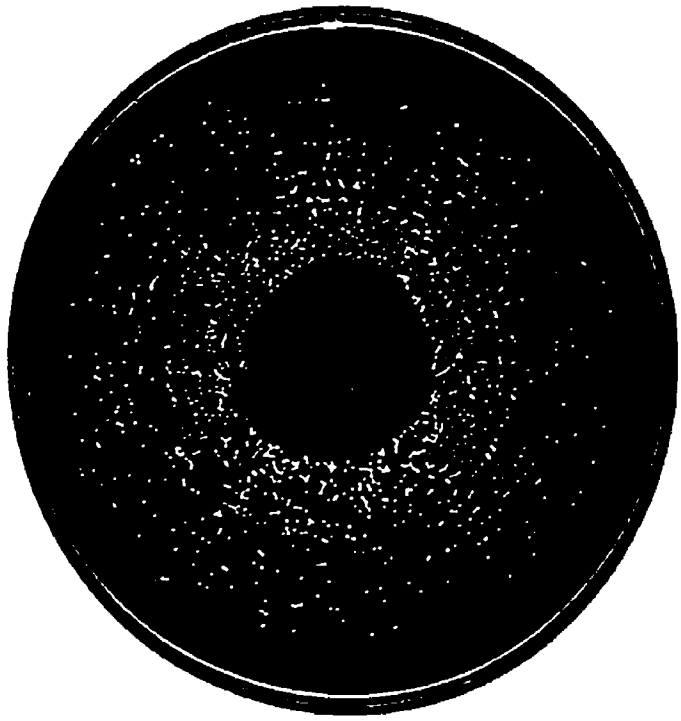


Figure 6. Visible growth of microorganisms after incubation by the spiral-plate method using Autoplater® 4000 model.

100 mm Spiral Counting Grid
(Model SCG-1701)

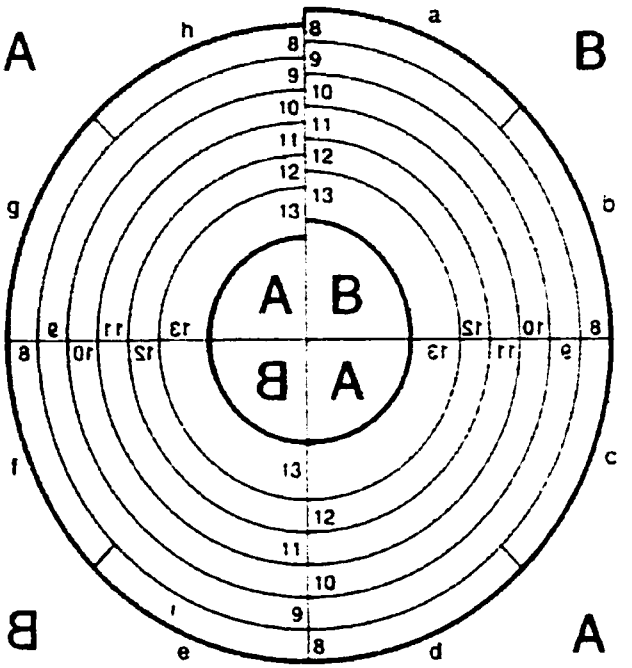


Figure 7. Pie-shaped grid used to count the number of microorganisms on a Petri dish by hand (Spiral Biotech, Inc., 1993).

3.2.6. Statistical Analysis

Data were analysed using JMP IN Statistical Discovery Software (version 3.2.1 for Windows). Water activity (a_w) and colour test data were analysed using Tukey-Kramer Honestly Significant Difference test where more than one sample mean was compared (Steel et al., 1997b). The differences between means for microbiology analysis of sampled coins and preliminary tests were analysed using the Tukey-Kramer Difference test and Student's t statistic test where two separate independent means were compared. The latter test is more sensitive than the Tukey-Kramer Difference test (Steel et al., 1997a and 1997b). The two multiple range comparison tests were used because JMP IN Statistical Discovery Software was unable to analyse the data using Duncan's Multiple Range test. Both tests were used because each represented extreme ends of multiple range comparison tests, Duncan's test being in the middle. Therefore, if both the Tukey-Kramer Difference test and Student's t statistic test showed the same results, one can conclude that Duncan's test would also have yielded the same result if it had been used.

CHAPTER 4

Results and Discussion

4.1. Microorganism Identification Tests

4.1.1. Morphology Test

Morphology was verified under the Zeiss phase contrast microscope to support the identification of strains used both in preliminary tests and meat trials. *Leuconostoc mesenteroides* ssp. *mesenteroides* cells were coccoid in shape, either in pairs or chains. These results are the same as described by Garvie (1986). *Lactobacillus sakei* was identified as straight rods with rounded ends, but at times was observed as slightly curved in shape. The cells occurred singly or in short chains and results matched the description by Kandler and Weiss (1986). Cells of *Lb. curvatus* occurred as bent rods with rounded ends. They were usually observed in pairs or short chains. Again, the characteristics were in agreement with the description by Kandler and Weiss (1986).

The five unknown microorganisms that were isolated from violet red bile glucose (VRBG) agar plates during meat trials were observed to be rod shaped cells that occurred singly or in pairs. Further tests were performed to help identify the unknown strains.

4.1.2. Gram Test by KOH

Gram test by KOH confirmed that *Lc. mesenteroides* ssp. *mesenteroides*, *Lb. sakei* and *Lb. curvatus* were Gram-positive bacteria since all three strains resulted in watery suspensions when 3% potassium hydroxide (KOH) was applied. The results correlated well with the findings reported by Gregersen (1978).

The unknown strains observed during the meat trials produced a viscous thread-like slime that followed the loop indicating that all were Gram-negative bacteria.

4.1.3. Gram Stain Test

For this work, *Lc. mesenteroides* ssp. *mesenteroides*, *Lb. sakei* and *Lb. curvatus* remained dark purple in colour, which were clearly seen under the microscope, indicating all three strains were Gram-positive bacteria.

The unknown strains found during the meat trials appeared pink in colour under the microscope, indicating the strains were Gram-negative bacteria.

4.1.4. Catalase Test

Catalase test was primarily used during the meat trials and tested on streptomycin thallous acetate actidione (STAA) medium. In general, only *Brochothrix thermosphacta* grow on this selective medium, however, it was found during this work that both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* also grew. When 3% hydrogen peroxide (H_2O_2) was applied to the colonies on STAA medium, only *B. thermosphacta* displayed gas bubbles indicating a positive reaction. The two lactic acid bacteria (LAB) strains used were catalase negative (absence of gas).

4.1.5. Motility Test

All unknown strains isolated from the meat trials showed no motility, indicating that the strains may not possess flagella.

4.1.6. API 20 E Test

The results of the five API 20 E tests indicated that the unknown strains were not from the same species, genus or family. One test yielded a poor fit with known biochemistry profiles and the identification was regarded as unreliable, therefore, the results were inconclusive. The remaining unknown strains resulted in a poor identification of *Klebsiella pneumoniae ozaenae*; a good identification of genus *Pantoea* (species not identified); an acceptable identification of genus *Citrobacter* (species not identified); and a poor identification of *Escherichia vulneris*.

It should be noted that the percentage of quantification of each unknown organism has been derived by the API 20 E product testing and therefore, may not be equivalent to or reflect conventional percentages. As well, substrate variations exist that can account for percentage differences. For example, the urease test found on API 20 E is a Ferguson formulation which is less sensitive than Christensen's formulation that is used widely in existing macrotechniques (BioMérieux Vitek, 1996). Therefore, words such as poor, good, and acceptable identifications are used instead of recording percentage of similarity when comparing unknown organisms to known standard organisms.

4.1.6.1. Summary

All four API 20 E strips did not result in excellent identifications; therefore, all unknown strains were not 100% conclusively identified. As one result indicated, an unknown organism was identified as *Pantoea*. This is a new genus that has been identified since the last Bergey's Manual of Systematic Bacteria was published in 1984. *Pantoea* is a Gram-negative, noncapsulated, nonsporing straight rod that is motile,

however, its motility was not seen in the motility test. It is also oxidase-negative and unable to grow at 44°C. It is found in plants, seeds and in soil, water, and human specimens. This genus has only two species known to date, which are *P. agglomerans* and *P. dispersa*. The former included *Enterobacter agglomerans*, *Erwinia herbicola*, and *E. milletiae* at one time (Jay, 1992).

The tests were not repeated due to time constraints. However, if the API 20 E experiments were repeated for all of the five unknown organisms, differences could have been explored. Of all the 20 microtubes containing dehydrated substrates, 9 carbohydrate microtubes did not match the API 20 E profile recognition system completely; therefore, this resulted in unreliable identifications of the unknown strains. Utilisation of the carbohydrates produces acid and lowers the pH within the microtube allowing the colour indicator to change from blue to yellow. In most cases, this change in colour was not apparent and a light lime green colour prevailed even after another 48 h incubation.

It was difficult to determine the identity of the unknown strains using only a few identification tests. More specific tests, for example ID 32 E from bioMérieux Inc., could be done in order to better determine the identity of the unknown strains; unfortunately, time did not allow for this.

4.2. Individual Resistance Tests

4.2.1. Inhibitory Factor Study

4.2.1.1. Eugenol and Liquid Smoke Alone

The results showed that the highest concentrations of liquid smoke (1000 ppm (v/v)) and eugenol (1000 ppm (v/v)) alone (Table 2) at 25°C were unable to prevent the

growth of bacteria in the test tubes after 48 h. Therefore, this indicates that the test concentrations used, the antimicrobials were ineffective at inhibiting growth of all three LAB strains at room temperature. The results are shown in Tables 15-17. It is interesting that Blaszyk and Holley (1998), in their experiments using microtitre plates, found that 1000 ppm (v/v) eugenol was able to prevent growth of *Lc. mesenteroides* at 18°C. Differences in incubation temperatures may have resulted in the different conclusions between Blaszyk and Holley (1998) experiment (at 18°C) and the present study (at 25°C); the present study being at a temperature in the optimal temperature range for these LAB to have favourable growth. They also found that *Lc. mesenteroides* was more sensitive to eugenol than *Lb. sakei* and *Lb. curvatus*. In their experiments ≤ 0.4% (w/v) sodium citrate was used as a chelator, which may have helped the inhibitory effect to occur. However, when eugenol was used for the current work without any other additions, it was found to be unable to inhibit all three LAB strains.

4.2.1.2. Eugenol, Liquid Smoke and Horseradish Oil Alone or in Combination

Each antimicrobial (1000 ppm (v/v) eugenol, 1000 ppm (v/v) liquid smoke, 120 ppm (v/v) horseradish oil) was tested against all three microorganisms at room temperature at different times up to 72 h (Table 3). It was found that at 24 h all three antimicrobials, individually, were able to reduce *Lc. mesenteroides* ssp. *mesenteorides* growth compared to that of the control. However, these particular antimicrobial concentrations were unable to inhibit the growth of *Lc. mesenteroides* ssp.

Table 15. Effect of various concentrations of liquid smoke and eugenol on *Lc. mesenteroides* ssp. *mesenteroides* at 25°C.

	Log_{10}CFU/ml	
Treatment/Time (hours)	0 hours	48 hours
Control*	4.00	9.33
700 ppm liquid smoke	4.00	9.30
800 ppm liquid smoke	4.00	9.31
900 ppm liquid smoke	4.00	9.29
1000 ppm liquid smoke	4.00	9.24
700 ppm eugenol	4.00	9.17
800 ppm eugenol	4.00	9.11
900 ppm eugenol	4.00	9.15
1000 ppm eugenol	4.00	9.14

* control consisted of MRS broth and target organism.

Table 16. Effect of various concentrations of liquid smoke and eugenol on *Lb. sakei* at 25°C.

	Log_{10}CFU/ml	
Treatment/Time (hours)	0 hours	48 hours
Control*	4.00	8.77
700 ppm liquid smoke	4.00	8.56
800 ppm liquid smoke	4.00	8.64
900 ppm liquid smoke	4.00	8.62
1000 ppm liquid smoke	4.00	8.82
700 ppm eugenol	4.00	8.64
800 ppm eugenol	4.00	8.69
900 ppm eugenol	4.00	8.58
1000 ppm eugenol	4.00	8.83

* control consisted of MRS broth and target organism.

Table 17. Effect of various concentrations of liquid smoke and eugenol on *Lb. curvatus* at 25°C.

	Log_{10}CFU/ml	
Treatment/Time (hours)	0 hours	48 hours
Control*	4.00	9.55
700 ppm liquid smoke	4.00	9.62
800 ppm liquid smoke	4.00	9.53
900 ppm liquid smoke	4.00	9.55
1000 ppm liquid smoke	4.00	9.51
700 ppm eugenol	4.00	9.29
800 ppm eugenol	4.00	9.28
900 ppm eugenol	4.00	9.33
1000 ppm eugenol	4.00	9.22

* control consisted of MRS broth and target organism.

growth compared to that of the control. However, these particular antimicrobial concentrations were unable to inhibit the growth of *Lc. mesenteroides* ssp. *mesenteroides* after 48 h. For both *L.b sakei* and *Lb. curvatus*, the antimicrobial concentrations had no effect. The results are shown in Tables 18-20. Overall the antimicrobials, at the specific concentration used were unable to inhibit the three target microorganisms.

Both eugenol and horseradish oil (Table 4) were tested alone or in combination against *Lc. mesenteroides* ssp. *mesenteroides* for 15 days at 8°C. The various concentrations of eugenol and horseradish oil used were unable to substantially inhibit the growth of this microorganism. Tables 21 and 22, and Figures 8 and 9 denote the results. However, the combination of 1000 ppm (v/v) eugenol and 100 ppm (v/v) horseradish oil was able to slightly reduce the growth of *Lc. mesenteroides* ssp. *mesenteroides*, but did not limit growth enough to have application in meat systems (Figure 9).

Liquid smoke (750 and 1000 ppm (v/v)) and horseradish oil (60, 80 and 100 ppm (v/v)) combinations (Table 5) were able to delay the growth of *Lc. mesenteroides* ssp. *mesenteroides* at 8°C during the 15 day testing period. However, growth was hampered only when liquid smoke was used at higher concentrations, 750 ppm (v/v) and 1000 ppm (v/v) when combined with horseradish oil (Figures 10-12). It was also interesting that neither liquid smoke (1000 ppm (v/v)) or horseradish oil (60, 80 and 100 ppm (v/v)) alone were unable to reduce the growth of the target organism until the two antimicrobials were combined with one another (Figures 13 and 8, respectively). Therefore it can be concluded that a combination of liquid smoke and horseradish oil are more effective against *Lc. mesenteroides* ssp. *mesenteroides* than when used separately.

Table 18. Effect of 1000 ppm (v/v) eugenol, 120 ppm (v/v) horseradish oil and 1000 ppm (v/v) liquid smoke on *Lc. mesenteroides* ssp. *mesenteroides* at 25°C.

	Log_{10}CFU/ml					
Treatment/Time (hours)	0	6	12	24	48	72
Control*	3.22	3.96	4.15	8.42	9.00	7.78
1000 ppm eugenol	3.35	4.03	5.03	5.99	9.10	7.82
1000 ppm liquid smoke	3.29	4.01	5.10	5.81	9.27	7.42
120 ppm horseradish oil	3.32	3.69	3.93	6.61	9.09	8.56

* control consisted of MRS broth and target organism.

Table 19. Effect of 1000 ppm (v/v) eugenol, 120 ppm (v/v) horseradish oil and 1000 ppm (v/v) liquid smoke on *Lb. sakei* at 25°C.

	Log_{10}CFU/ml					
Treatment/Time (hours)	0	6	12	24	48	72
Control*	2.44	3.74	4.87	5.42	8.03	8.37
1000 ppm eugenol	2.45	3.76	4.31	5.48	8.26	8.08
1000 ppm liquid smoke	2.62	3.82	4.12	6.08	8.41	8.12
120 ppm horseradish oil	2.46	2.97	3.36	5.42	8.26	7.60

* control consisted of MRS broth and target organism.

Table 20. Effect of 1000 ppm (v/v) eugenol, 120 ppm (v/v) horseradish oil and 1000 ppm (v/v) liquid smoke on *Lb. curvatus* at 25°C.

	Log_{10}CFU/ml					
Treatment/Time (hours)	0	6	12	24	48	72
Control*	3.46	4.91	4.14	3.12	9.21	9.46
1000 ppm eugenol	3.41	4.94	4.41	6.76	8.89	9.14
1000 ppm liquid smoke	3.51	4.69	4.12	5.15	8.94	9.17
120 ppm horseradish oil	3.52	3.64	3.68	3.67	7.30	7.30

*control consisted of MRS broth and target organism.

Table 21. Effect of 500 ppm (v/v) eugenol and several concentrations of horseradish oil on *Lc. mesenteroides* ssp. *mesenteroides* at 8°C.

	Log_{10}CFU/ml						
Treatment/Day(s)	0	0.5	1	2	7	12	15
Control*	3.35	4.47	6.19	5.20	7.37	8.92	8.93
500 ppm eugenol + 60 ppm horseradish oil	3.38	4.24	5.12	5.49	8.10	9.47	8.72
500 ppm eugenol + 80 ppm horseradish oil	3.39	4.21	4.73	5.33	7.59	8.80	8.70
500 ppm eugenol + 100 ppm horseradish oil	3.33	4.22	4.83	5.53	7.10	8.29	8.42

*control consisted of MRS broth and target organism.

Table 22. Effect of 750 ppm (v/v) eugenol and several concentrations of horseradish oil on *Lc. mesenteroides* ssp. *mesenteroides* at 8°C.

	Log_{10}CFU/ml						
Treatment/Day(s)	0	0.5	1	2	7	12	15
Control*	3.52	4.47	6.19	5.20	7.37	8.92	8.93
750 ppm eugenol + 60 ppm horseradish oil	3.54	4.30	5.41	5.48	8.11	8.82	8.65
750 ppm eugenol + 80 ppm horseradish oil	3.48	4.24	5.07	5.50	7.44	8.64	8.65
750 ppm eugenol + 100 ppm horseradish oil	3.46	4.16	5.13	5.46	7.18	8.54	8.67

*control consisted of MRS broth and target organism.

Figure 8: Effect of various concentrations of horseradish oil on *Lc. mesenteroides* ssp. *mesenteroides* at 8°C.

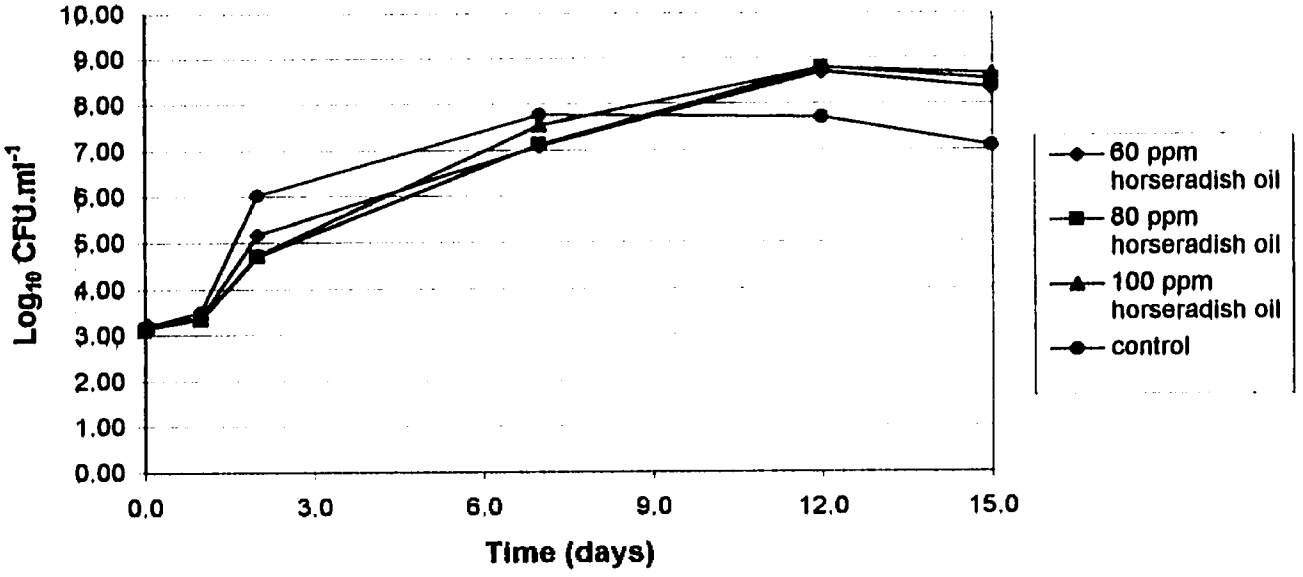


Figure 9: Effect of 1000 ppm (v/v) eugenol and horseradish oil on *Lc. mesenteroides* ssp. *mesenteroides* at 8^{0}C.

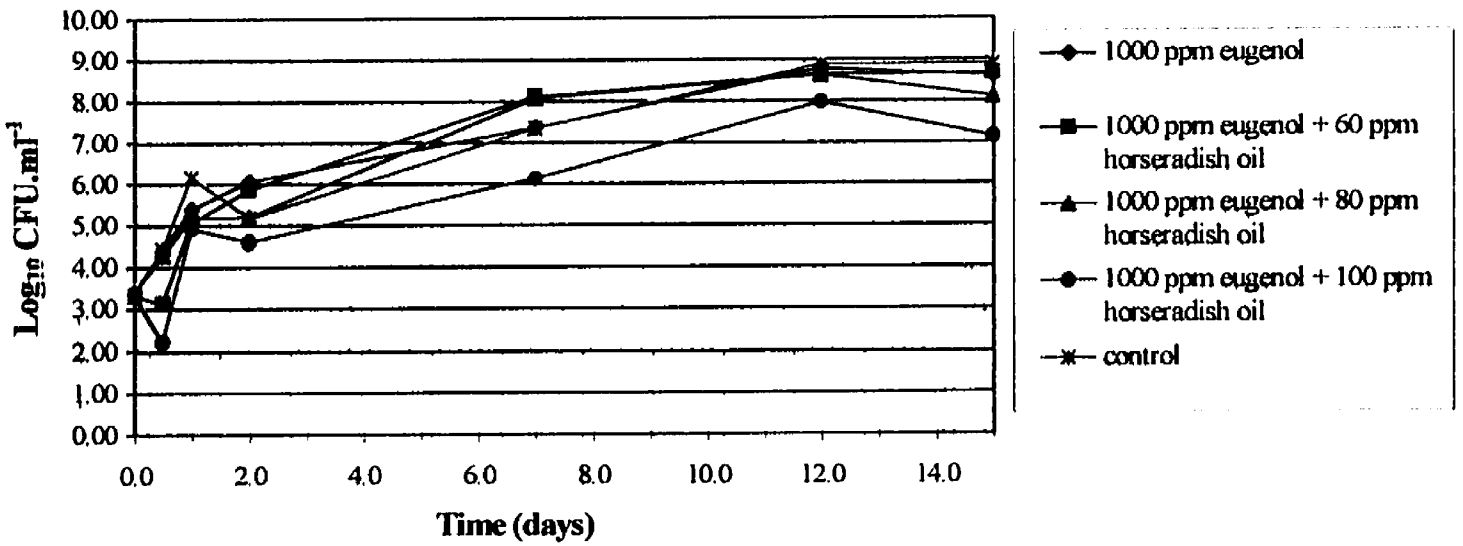


Figure 10: Effect of 500 ppm (v/v) liquid smoke and various concentrations of horseradish oil on *Lc. mesenteroides* ssp. *mesenteroides* at 8°C.

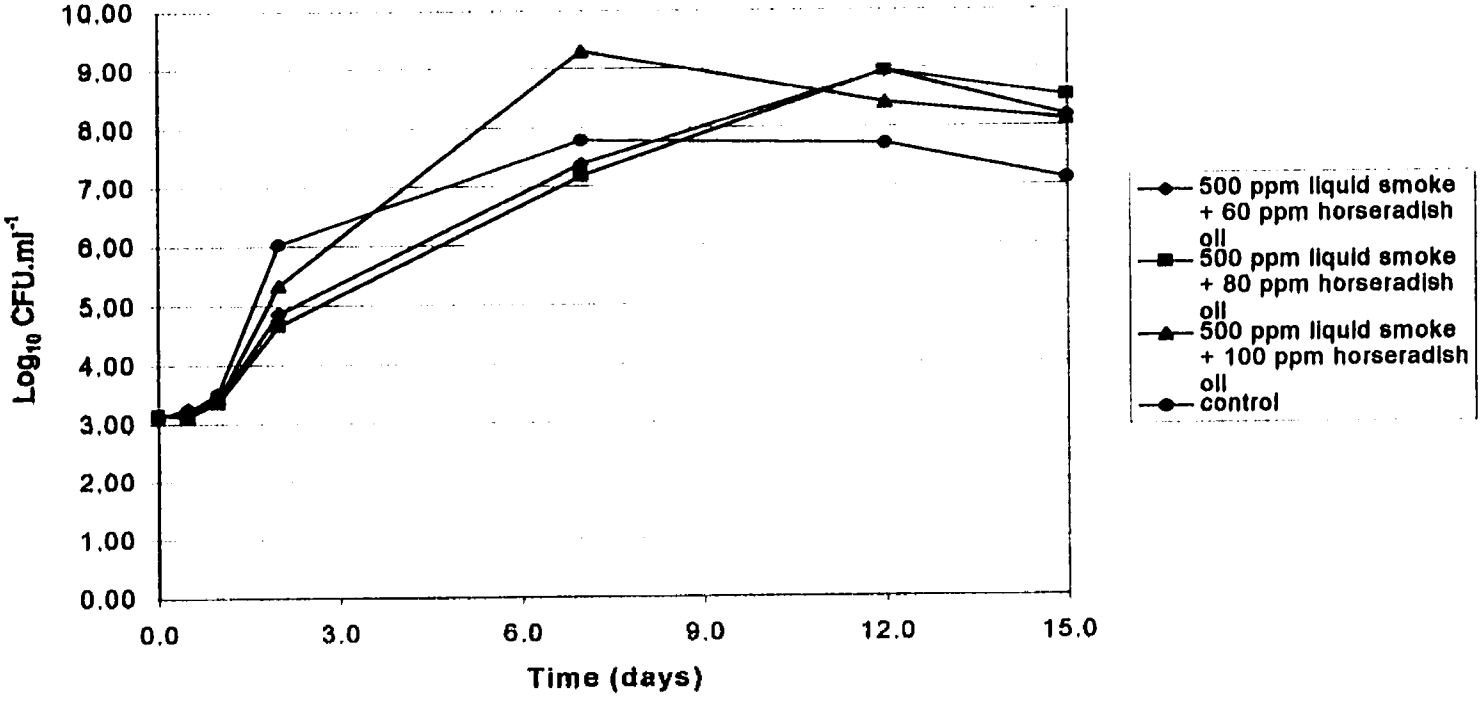


Figure 11: Effect of 750 ppm (v/v) liquid smoke and various concentrations of horseradish oil on *Lc. mesenteroides* ssp. *mesenteroides* at 8°C.

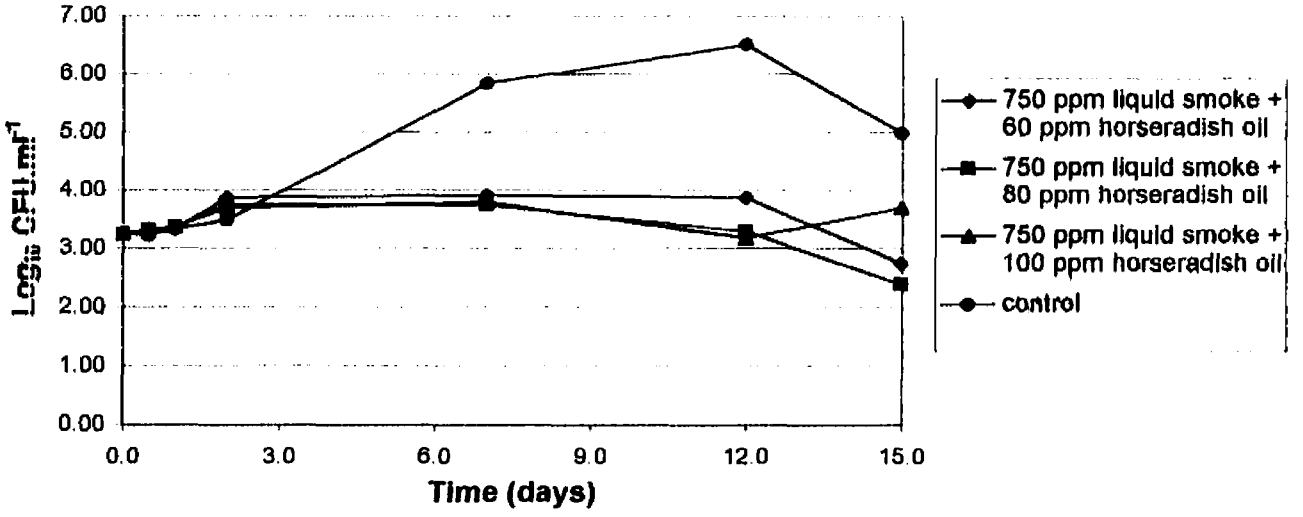


Figure 12: Effect of 1000 ppm (v/v) liquid smoke and various concentrations of horseradish oil on *Lc. mesenteroides* ssp. *mesenteroides* at 8°C.

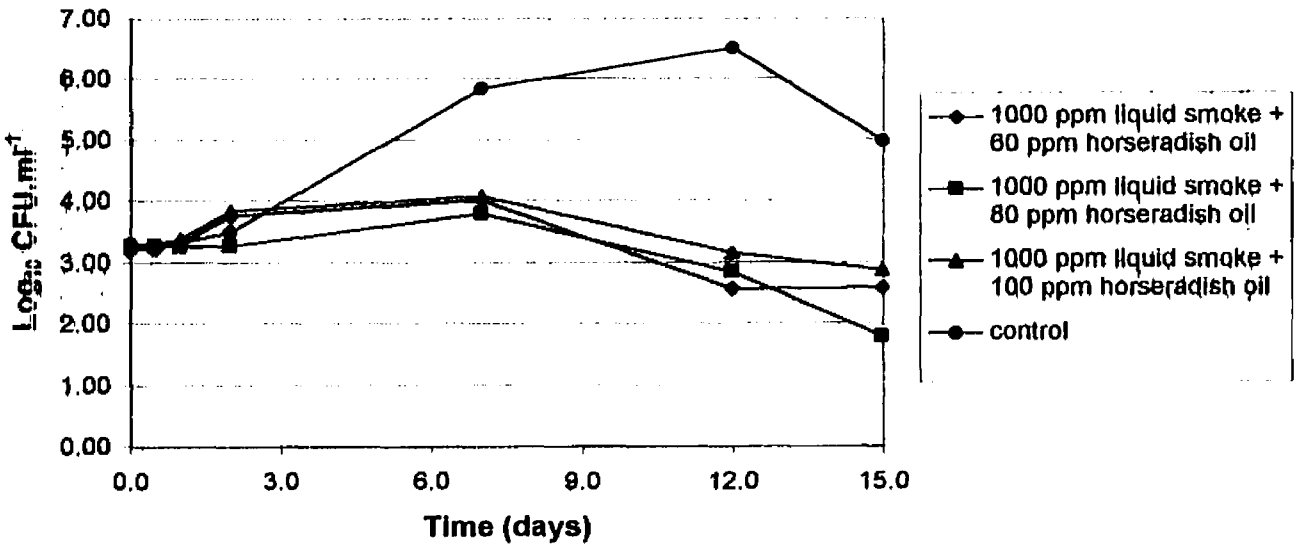
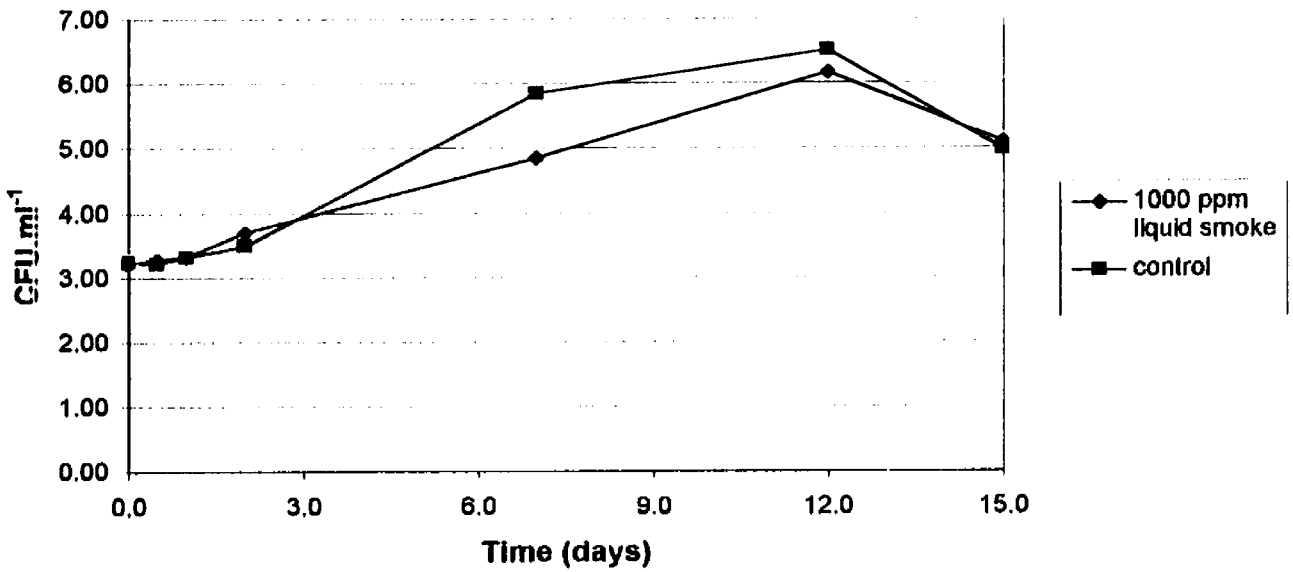


Figure 13: Effect of 1000 ppm (v/v) liquid smoke on *Lc. mesenteroides* ssp. *mesenteroides* at 8°C.



4.2.1.3. Combinations of Eugenol, Liquid Smoke, Horseradish Oil, Lauricidin® and Polyphosphoric Acid

The combination of eugenol, horseradish oil (60 to 100 ppm (v/v)), 250 ppm (w/v) Lauricidin® and 4000 ppm (v/v) polyphosphoric acid (Table 6) retarded the growth of *Lc. mesenteroides* ssp. *mesenteroides* compared to the control, which contained only the target organisms and no antimicrobials (Table 23). The delay in growth probably was due to polyphosphoric acid, since it has a pH ~4.81 at 25°C when combined with MRS broth. The growth of the organism was either halted or slowed down tremendously in the presence of the acid. Since polyphosphoric acid had an extreme effect on *Lc. mesenteroides* ssp. *mesenteroides* the other antimicrobials most likely had no effect on the growth and consequently the outcome of the results. Unfortuantely, the pH was not measured during this experiment.

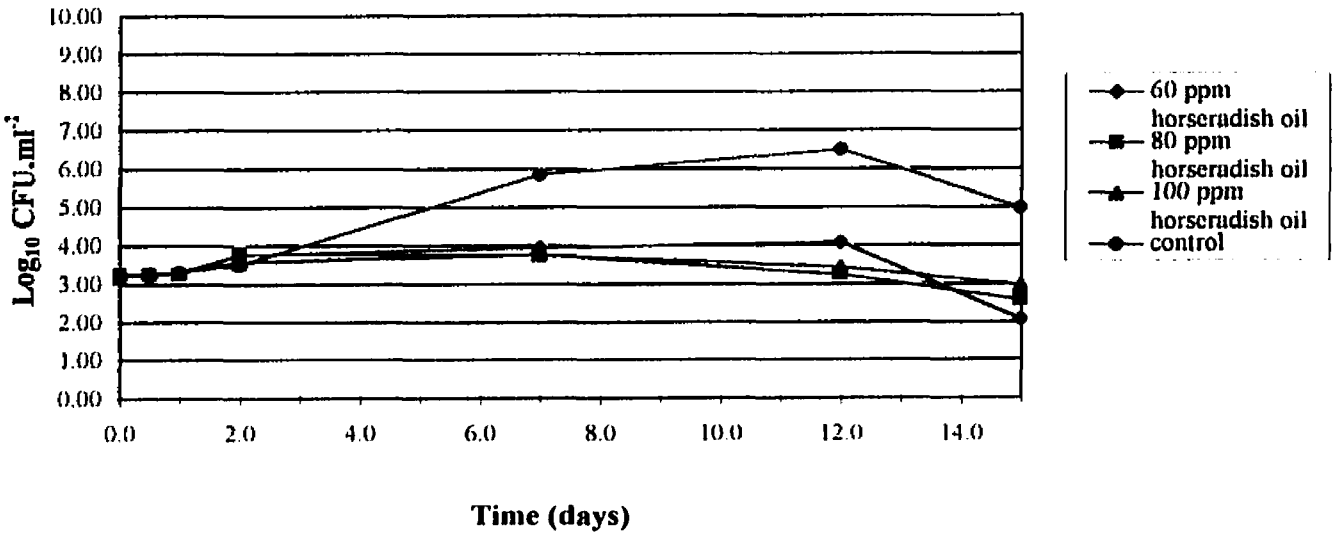
Combinations of liquid smoke and horseradish oil alone or in combination supplemented with 250 ppm (w/v) Lauricidin® and 4000 ppm (v/v) polyphosphoric acid (Table 7) were able to inhibit the growth of *Lc. mesenteroides* ssp. *mesenteroides*. These results are clearly shown in Figures 14-17. However, 1000 ppm (v/v) liquid smoke alone or combined with 250 ppm (w/v) Lauricidin® and 0.4% (v/v) polyphosphoric acid was unable to inhibit growth (Figure 17). In fact, this combination allowed a steady increase in growth of bacteria. This suggests that liquid smoke, when combined with the other agents was not as effective as horseradish oil in preventing the growth of *Lc. mesenteroides* ssp. *mesenteroides*.

Table 23. Effect of various concentrations of eugenol, horseradish oil, 250 ppm (w/v) Lauricidin^{R} and 0.4% (v/v) polyphosphoric acid on *Lc. mesenteroides* ssp. *mesenteroides* at 8°C.

	Log_{10}CFU/ml					
Treatment(s)/Day(s)	0	0.5	1	2	12	15
Control*	3.44	4.39	6.82	5.50	8.97	7.50
60 ppm horseradish oil	3.21	3.31	4.38	3.31	5.52	3.60
80 ppm horseradish oil	3.21	3.33	3.22	3.23	5.52	3.73
100 ppm horseradish oil	1.52	2.93	4.25	3.19	5.42	3.78
500 ppm eugenol + 60 ppm horseradish oil	3.32	3.32	3.22	3.15	5.73	3.60
500 ppm eugenol + 80 ppm horseradish oil	3.36	3.32	3.23	3.17	5.67	3.67
500 ppm eugenol + 100 ppm horseradish oil	3.36	3.32	3.34	3.25	5.67	3.37
750 ppm eugenol + 60 ppm horseradish oil	1.67	2.08	3.00	2.59	5.42	3.73
750 ppm eugenol + 80 ppm horseradish oil	3.19	1.60	3.00	2.48	5.60	3.60
750 ppm eugenol + 100 ppm horseradish oil	2.12	1.30	1.52	2.65	5.52	3.60
1000 ppm eugenol	2.05	3.19	1.48	2.81	5.42	3.60
1000 ppm eugenol + 60 ppm horseradish oil	1.86	1.12	2.95	2.08	5.82	3.60
1000 ppm eugenol + 80 ppm horseradish oil	1.30	1.52	0.00	3.00	5.78	3.60
1000 ppm eugenol + 100 ppm horseradish oil	0.00	1.87	1.43	3.00	5.67	3.60

*control consisted of MRS broth and target organism.

Figure 14: Effect of horseradish oil supplemented with 0.4% (v/v) polyphosphoric acid and 250 ppm (w/v) Lauricidin^R on *Lc. mesenteroides* ssp. *mesenteroides* at 8^{0}C.



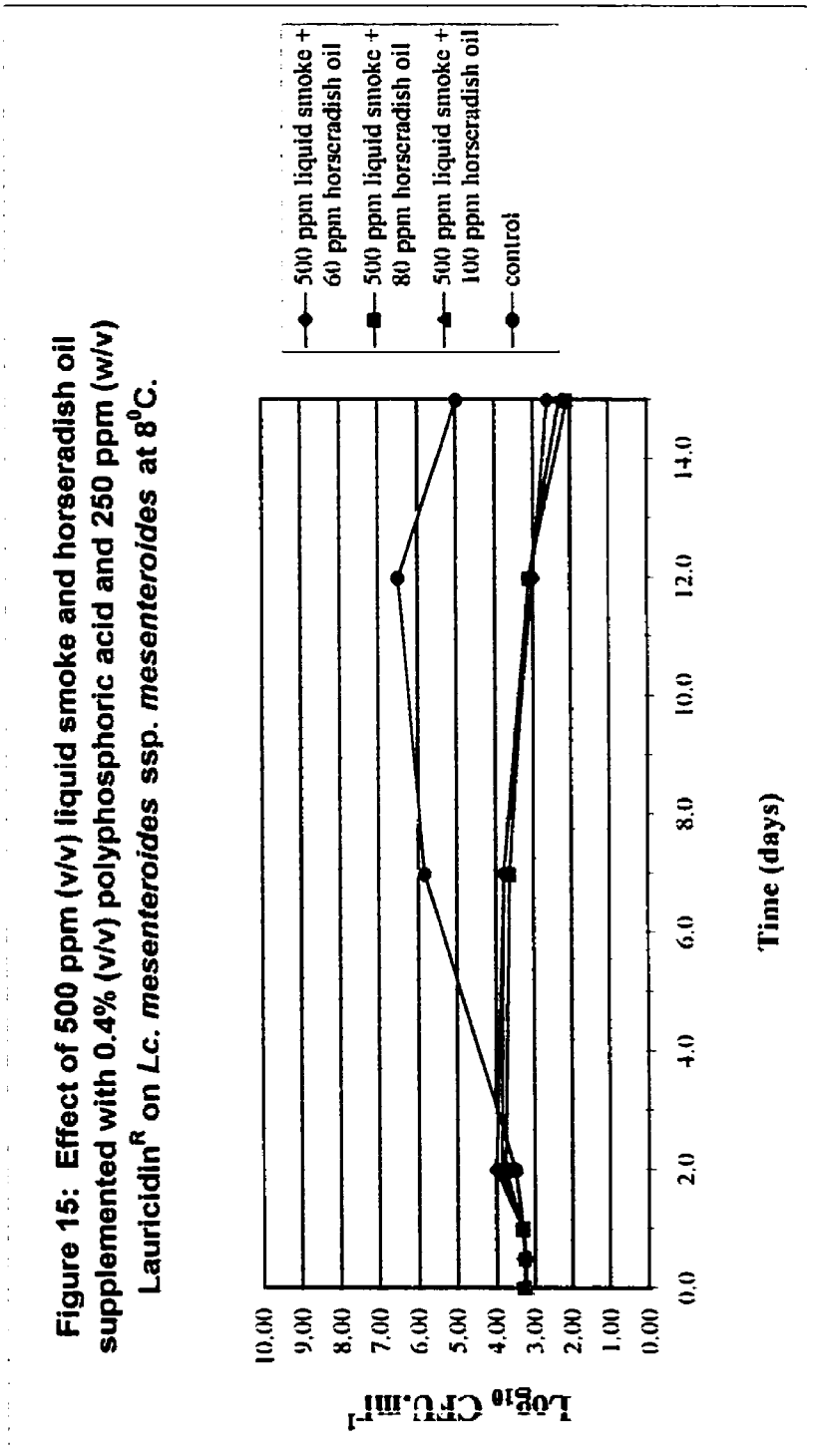


Figure 15: Effect of 500 ppm (v/v) liquid smoke and horseradish oil supplemented with 0.4% (v/v) polyphosphoric acid and 250 ppm (w/v) Lauricidin^R on *Lc. mesenteroides* ssp. *mesenteroides* at 8^{0}C.

Figure 16: Effect of 750 ppm (v/v) liquid smoke and horseradish oil supplemented with 0.4% (v/v) polyphosphoric acid and 250 ppm (w/v) Lauricidin^R on *Lc. mesenteroides* ssp. *mesenteroides* at 8^{0}C.

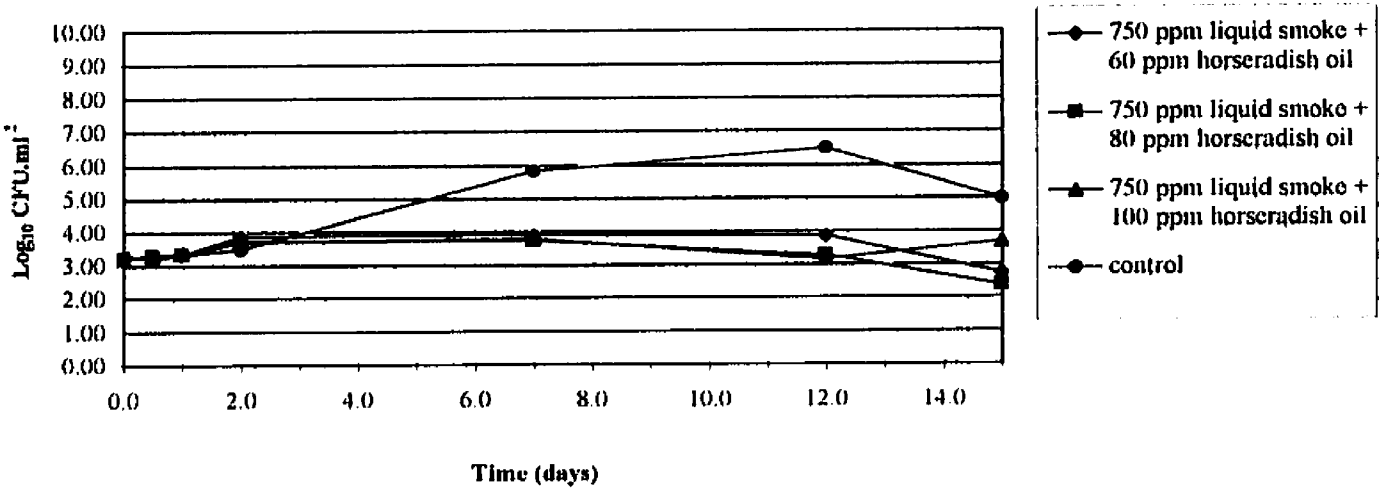
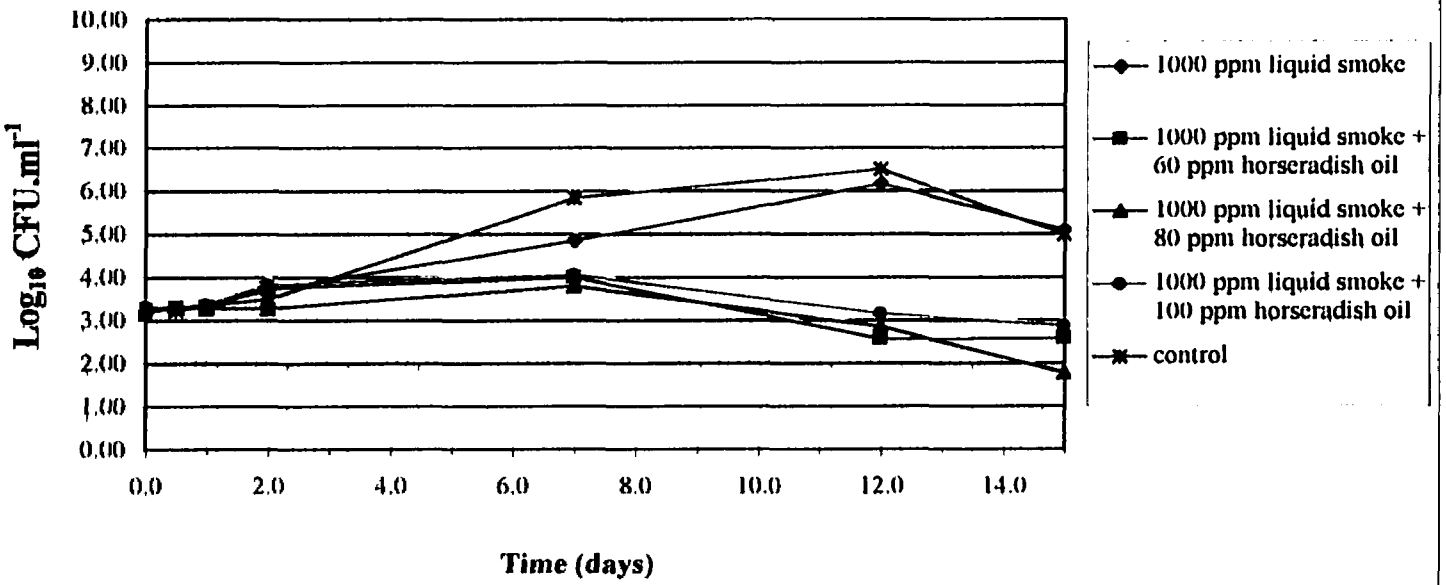


Figure 17: Effect of 1000 ppm (v/v) liquid smoke and horseradish oil supplemented with 0.4% (v/v) polyphosphoric acid and 250 ppm (w/v) Lauricidin^{R} on *Lc. mesenteroides* ssp. *mesenteroides* at 8^{0}C.



4.2.1.4. Lauricidin®, disodium pyrophosphate, potassium lactate and Alta® 2341 Alone or in Combination

The use of Lauricidin®, disodium pyrophosphate, potassium lactate an Alta® 2341 alone or in combination supplemented with 2.5% (w/v) NaCl and 100 ppm (w/v) $NaNO_2$ (Table 8) failed to inhibt both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* during the 14 day test period (Figures 18-25). It should be noted that both NaCl and $NaNO_2$ were added only to mimic the conditions found in cured meats.

The pH declined steadily with time pH ~6.4 to ~4.5. The increase numbers of both target organisms can explain this phenomenon. As the target organisms grew, they produced lactic acid as a by-product, which in turn decreased the pH of the broth. This decline in pH allows LAB to become dominant because most other spoilage organisms can not survive acidic environments.

4.2.1.5. Summary

The inhibitory factor tests showed that combinations of antimicrobials mixed with polyphosphoric acid and Lauricidin® were able to inhibit the growth of *Lc. mesenteroides* ssp. *mesenteroides* at 8°C (Table 23 and Figures 14-17). The inability of *Lc. mesenteroides* ssp. *mesenteroides* to grow probably resulted from the addition of polyphosphoric acid, which dropped the pH of the MRS broth from pH 6.5 to pH ~4.8. A more highly buffered system may have prevented the decrease in pH.

Liquid smoke (750 ppm and 1000 ppm (v/v)) plus horseradish oil (60-100 ppm (v/v)) effectively inhibited *Lc. mesenteroides* ssp. *mesenteroides* when combined (Figures 11 and 12), but were not successful alone in delaying the growth of the target

Figure 18: Effect of 1000 ppm (v/v) liquid smoke and horseradish oil supplemented with 0.4% (v/v) polyphosphoric acid and 250 ppm (w/v) Lauricidin^R on *Lc. mesenteroides* ssp. *mesenteroides* at 8^{0}C.

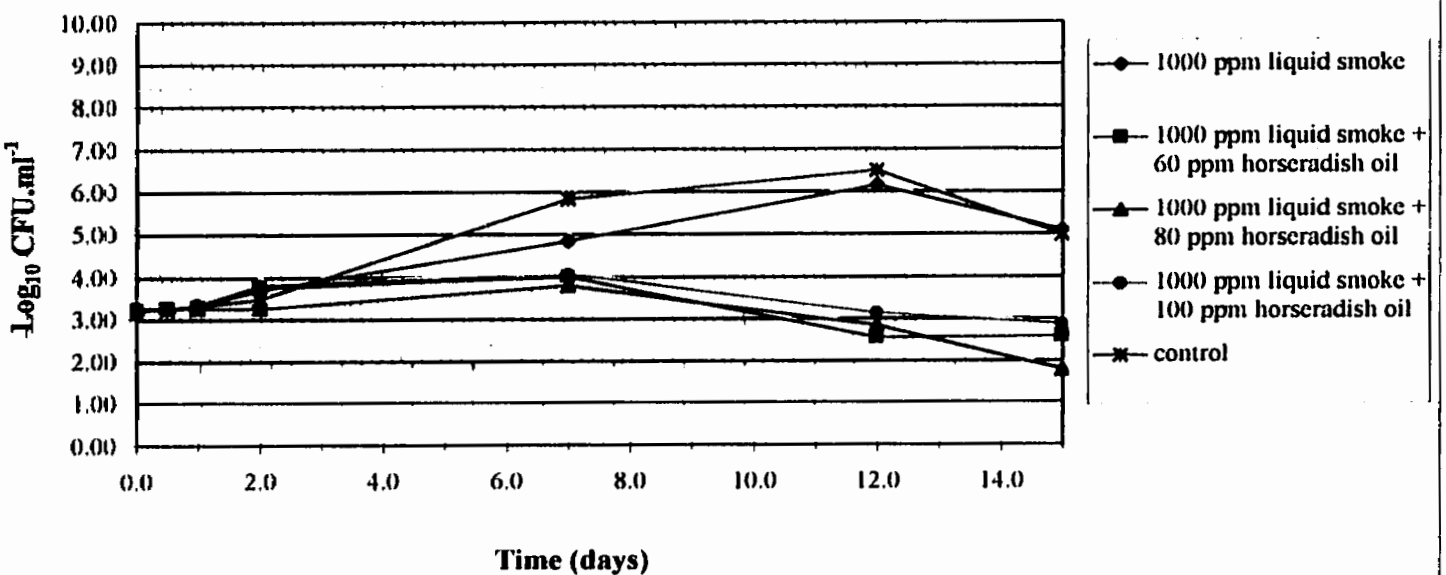


Figure 19: Effect of 250 ppm (w/v) Lauricidin^R and 0.4% (w/v) disodium pyrophosphate alone and in combination against *Lb. sakei* at 8°C.

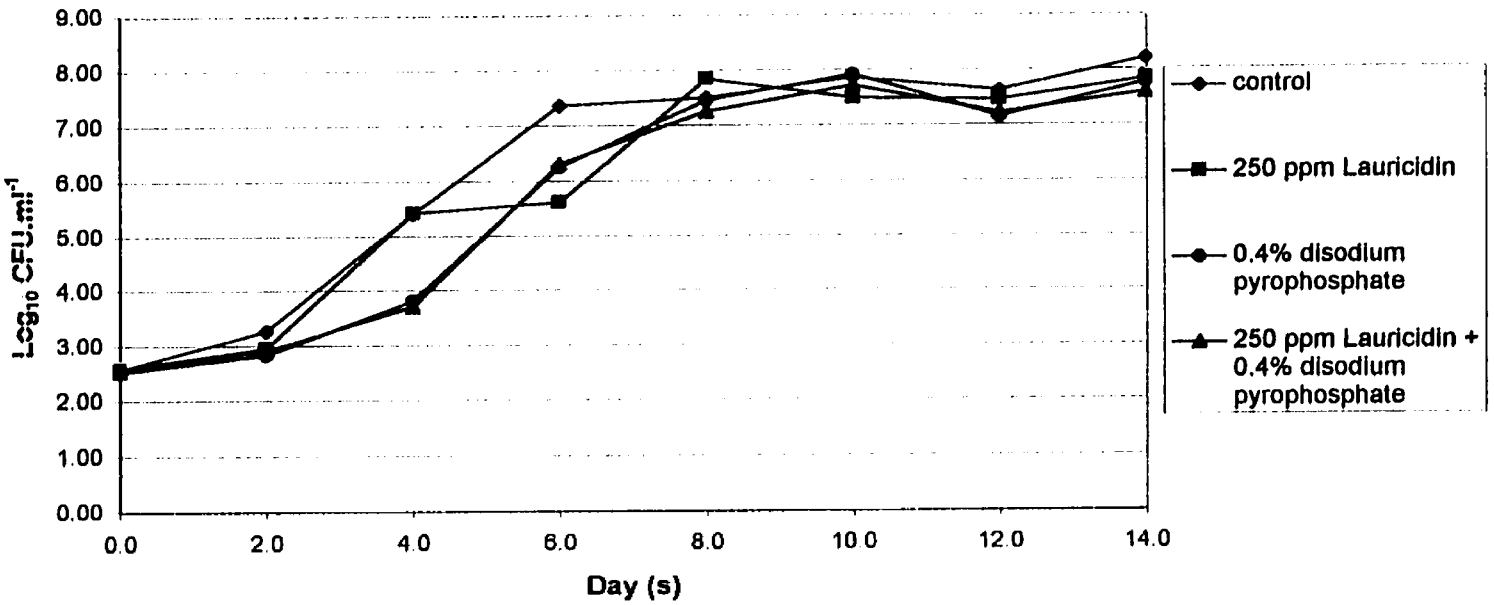


Figure 20: Effect of 500 ppm (w/v) Lauricidin^R and 0.4% (w/v) disodium pyrophosphate alone and in combination against *Lc. mesenteroides* ssp. *mesenteroides* at 8°C.

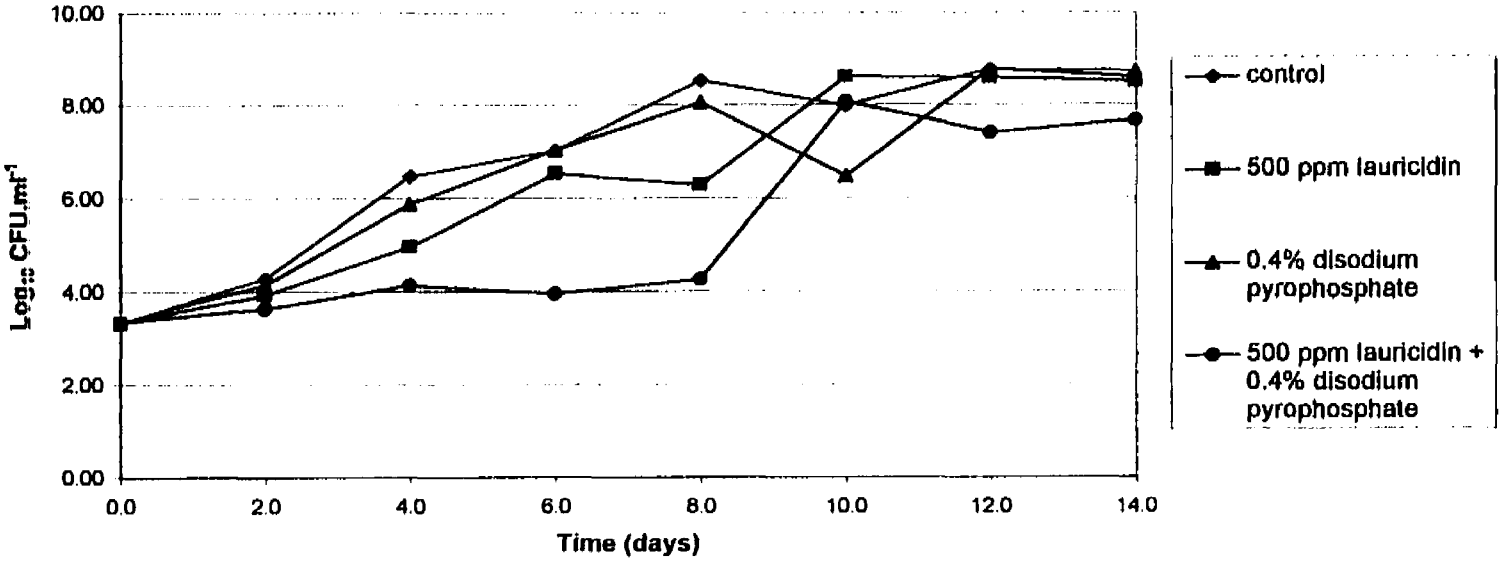


Figure 21: Effect of 500 ppm (w/v) Lauricidin^R and 0.4% (w/v) disodium pyrophosphate alone and in combination against *Lb. sakei* at 8°C.

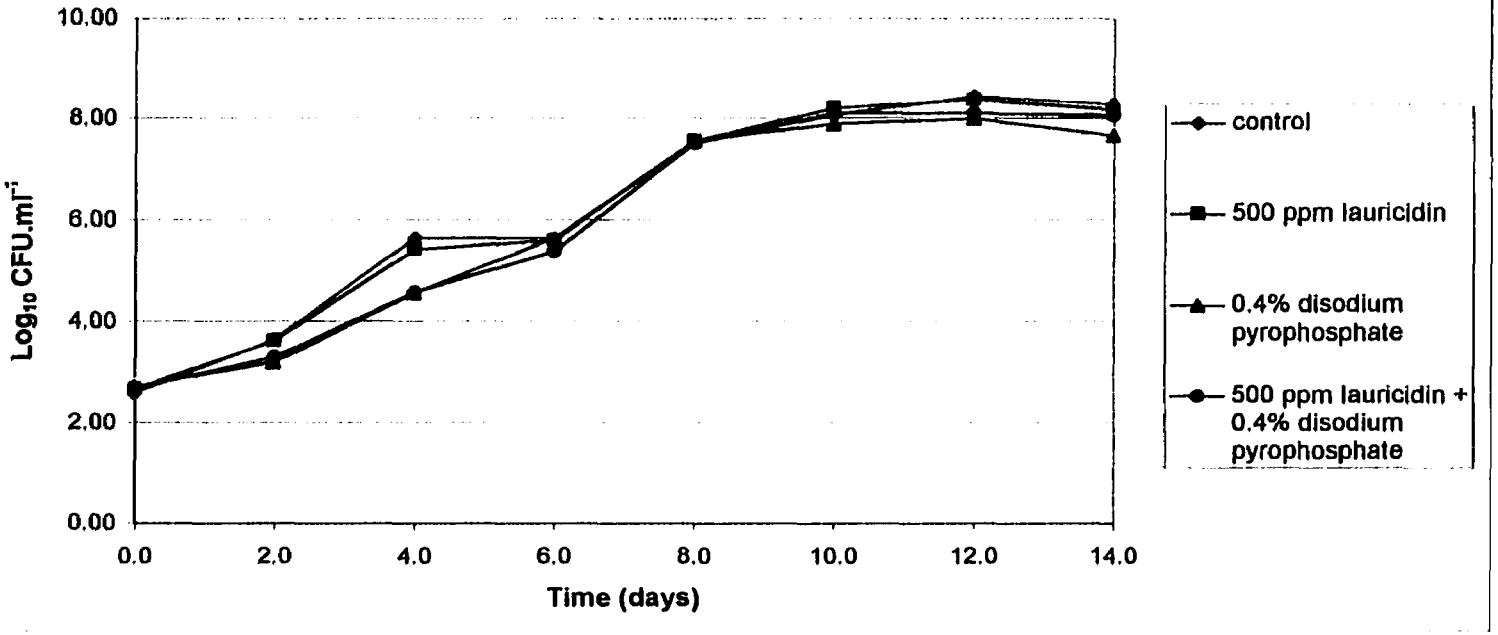
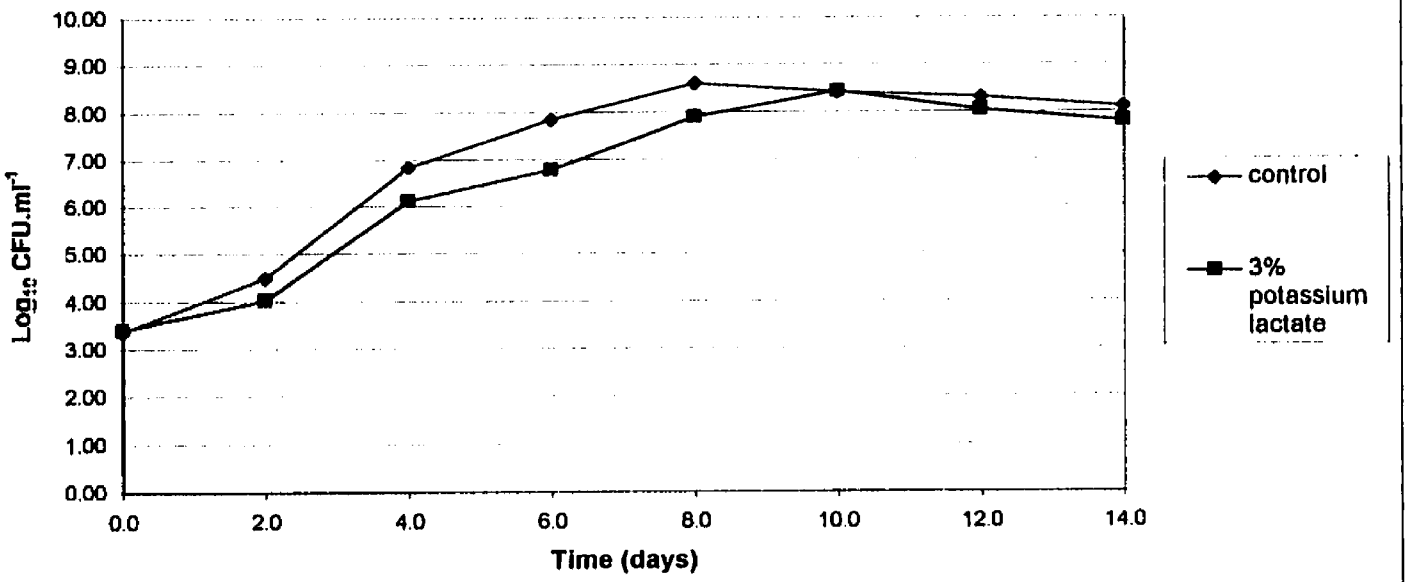


Figure 22: Effect of 3% (v/v) potassium lactate on *Lc. mesenteroides* ssp. *mesenteroides* at 8°C.



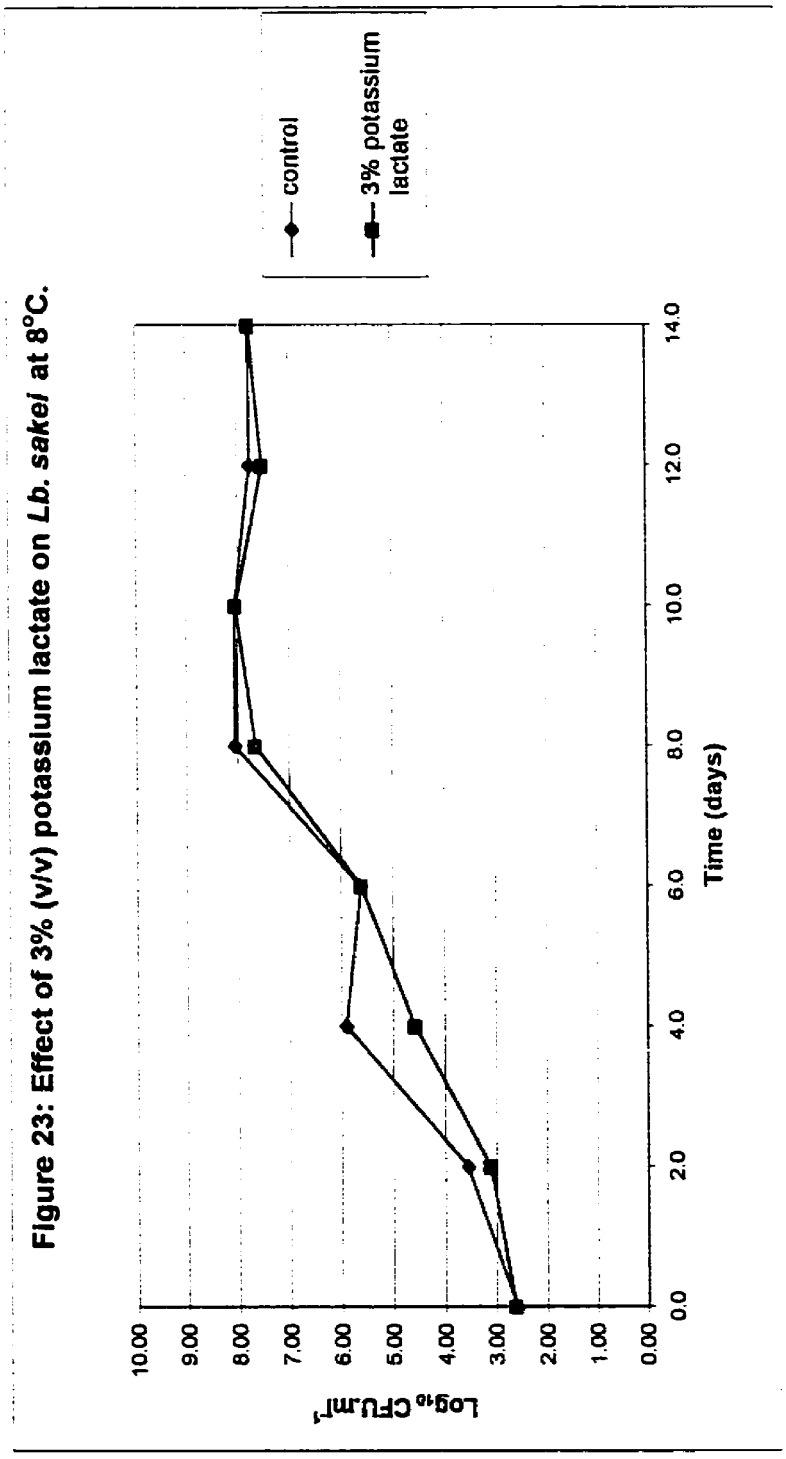
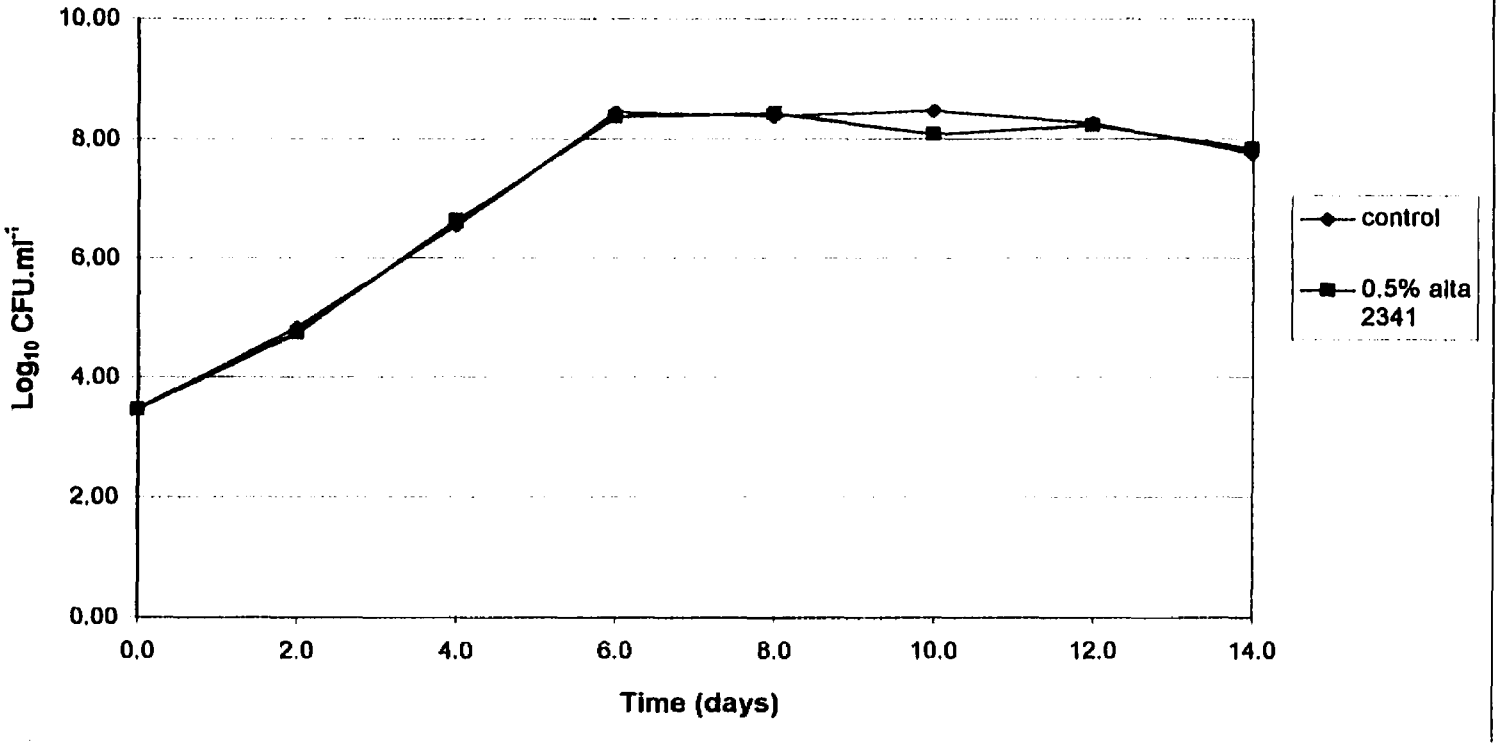


Figure 23: Effect of 3% (v/v) potassium lactate on *Lb. sakei* at 8°C.

Figure 24: Effect of 0.5% (w/v) Alta^{R} 2341 on *Lc. mesenteroides* ssp. *mesenteroides* at 8°C.



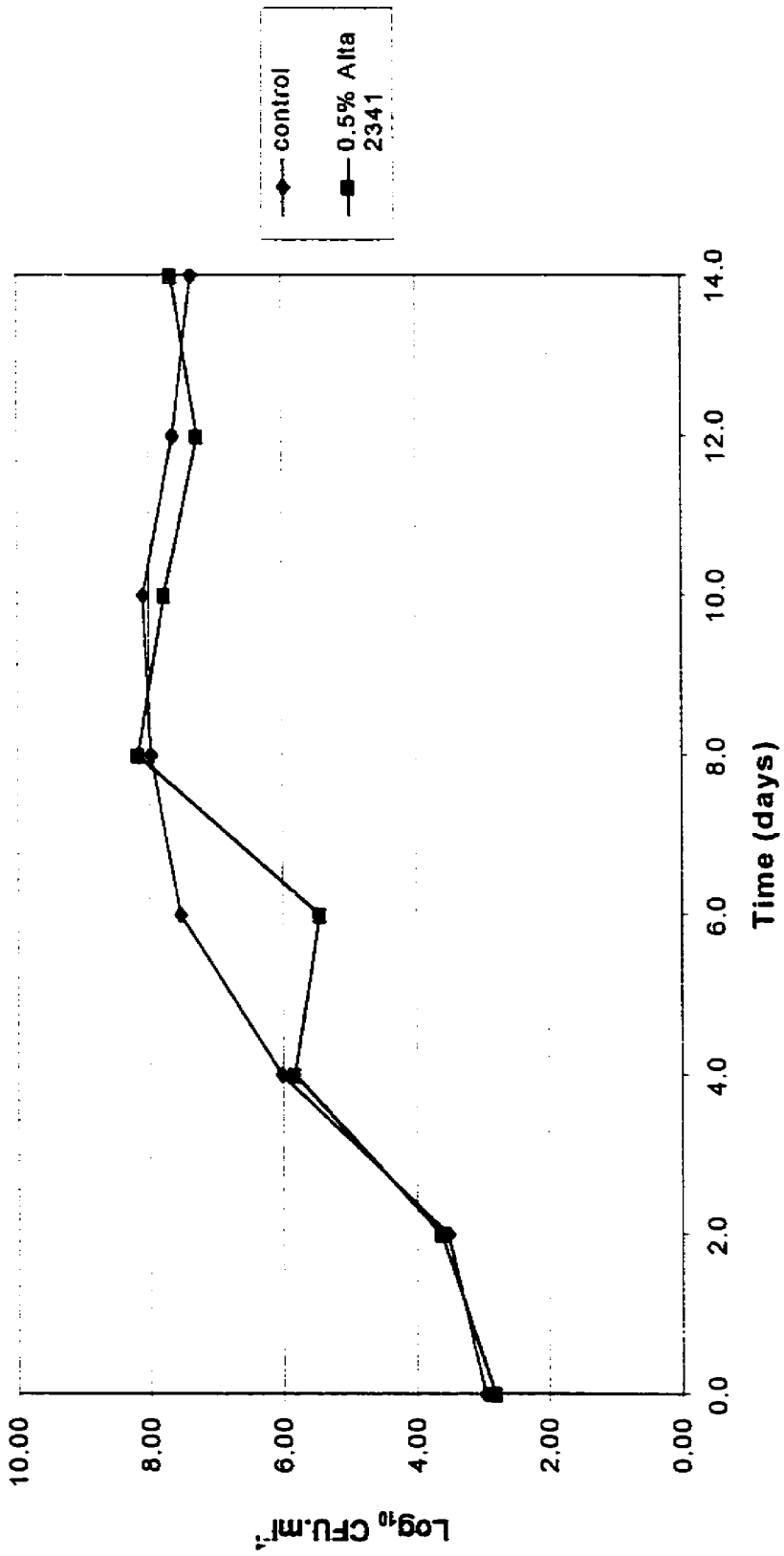


Figure 25: Effect of 0.5% (w/v) Alta^{R} 2341 on *Lb. sakei* at 8°C.

organism (Figure 13 and 8, respectively). The possible explanation for the inability of liquid smoke to inhibit the target organisms is that perhaps that there was not a high enough concentration of isoeugenol and eugenol in Aro-Smoke P-50 and when added to the MRS broth, it was further diluted below the inhibitory threshold (Rozum and Maurer, 1997). It should be noted that MRS was reconstituted using distilled water and because of their limited solubility the isoeugenol and eugenol (both being oils on their own) inhibitory effects may have been further reduced (Wendorff and Wee, 1997). Perhaps, liquid smoke would be more successful in delaying the growth of target microorganism(s) if applied to a meat model system. Eugenol, alone or as a component of liquid smoke, reacts with the cellular membrane of the target organism and impairs both the cell membrane's function and integrity (Raccach, 1984). A possible explanation for the inhibitory action of eugenol is that it inactivates enzymes and/or genetic function within the cell (Branen et al., 1980). This may in part explain the inhibitory action of liquid smoke towards microorganisms but other phenolic inhibitors are also present in commercial liquid smoke preparations. Researchers such as Faith et al. (1992) and Messina et al. (1988) have found success with liquid smoke in controlling microbial growth of *L. monocytogenes* in meat model systems. However, neither authors studied the mode of action of liquid smoke in their test products. The mechanism(s) by which AIT inhibits microorganisms are not well known. To date, there is no evidence of a single site of attack by AIT and therefore it has been described as a nonspecific inhibitor (Delaquis and Mazza, 1995). The literature states that AIT is known to react with free amino groups and disulfide bonds of proteins, and that it inhibits specific carriers in the electron transport chain and acts as an uncoupler of oxidative phosphorylation (Delaquis

and Sholberg, 1997). With this in mind, horseradish oil could retard the growth of a broad range of microorganisms. Although both horseradish oil (AIT) and liquid smoke were unable to inhibit the target organism alone, their effectiveness together was promising. With liquid smoke disrupting the cell membrane and inactivating enzymes, and horseradish oil reacting with a number of nonspecific sites the possibility of their success together outweighed their ability to inhibit the microorganism alone. The idea of using two or more antimicrobial components was proposed by Kabara (1993). He stated that multiple effects of several natural antimicrobial agents could create an undesirable environment for a broad spectrum of microrganisms, and these combinations were termed "preservative systems". This may explain the effectiveness of the combined horseradish oil and liquid smoke towards *Lc. mesenteroides* ssp. *mesenteroides* in the liquid medium.

Horseradish oil alone (Tables 18-20) was not effective against any of the three LAB strains. Ward et al. (1998) also found that *Lb. sakei* was only weakly inhibited by horseradish distillate at the highest concentration tested on agar surfaces (20 000 nL/L air). There are several possibilities why horseradish oil was not effective. For instance, Ohta et al. (1995a and 1995b) found that AIT was readily broken down in aqueous solutions especially at high water content. This decomposition is through nucleophilic attack by water on the isothiocyanate group of AIT in aqueous solution. These researchers also stated that high temperature affects the AIT decomposition greatly in aqueous solutions. This was seen in the present tests where incubation was conducted at room temperature. For example, Table 18-20 show at 120 ppm (v/v), horseradish oil was unable to inhibit *Lc. mesenteroides* ssp. *mesenteroides*, *Lb. sakei* and *Lb. curvatus* at 25°C. Several researchers have pointed out that AIT is more effective in the vapour

phase than in solution (Ward et al., 1998; Isshiki et al., 1992). Therefore, use of an aqueous system in the present study may have reduced the antimicrobial effectiveness of AIT.

In general, Gram-positive bacteria are more sensitive to spices and spice oils than Gram-negative bacteria, with the LAB being the most resistant among Gram-positives (Shelef, 1983). Zaika et al. (1983) stated that the mechanisms by which spices and spice oils inhibit microorganisms are unclear. They suggested that chemically unrelated spices and spice oils may have different mechanisms of inhibitory activity. For example, the inhibitory mechanisms for thyme, oregano, rosemary and sage may in fact be similar, because the resistance developed by some LAB to one spice was followed by resistance to the other three. In another paper by Zaika (1988), she stated that the active components of spice antimicrobials are generally only slightly soluble in aqueous systems, such as liquid bacterial growth medium. This may have been true in the case of inhibitory factor study test where eugenol, tested alone, (Tables 15-20) was unable to inhibit the target microorganisms. It was anticipated that the extent of inhibition by oils, such as eugenol, might be due to the presence of phenolic OH groups. Farag et al. (1989) stated that the OH group is quite reactive and can form hydrogen bonds with active sites of target enzymes. Other researchers have mentioned this theory as well. For instance, Raccach (1984) believed that the phenolic antioxidants react with the cellular membrane damaging both the functional capability and structure of the microorganism.

Blaszyk and Holley (1998) used microtitre plates and found that 100 and 250 ppm monolaurin (Lauricidin®) was able to strongly inhibit *Lc. mesenteroides*, but was unable to delay the growth of both *Lb. sakei* and *Lb. curvatus* at 7°C and 18°C. Other

researchers have also stated that monolaurin (Lauricidin®) has the ability to delay growth of spoilage and pathogenic bacteria that are associated with food (Razavi-Rohani and Griffiths, 1994; Oh and Marshall, 1993, 1994 and 1995; Wang and Johnson, 1992; Baker et al., 1982). Unfortunately in the inhibitory factor study tests, 250 ppm and 500 ppm (w/v) Lauricidin® alone were not able substantially to delay the growth of *Lc. mesenteroides* ssp. *mesenteorides* and *Lb. sakei* at 8°C over a 14 day testing period (Figures 19-22). However, when 500 ppm (w/v) Lauricidin® was combined with 0.4% (w/v) disodium pyrophosphate (Figure 21) *Lc. mesenteroides* ssp. *mesenteroides* was inhibited until day 10 of incubation at 8°C. This result is in agreement with Razavi-Rohani and Griffiths (1994) where they found that monolaurin showed antibacterial activity against a variety of bacteria when combined with a chelating agent and incubated at 37 or 25°C for 24 h using a spiral gradient end point test. Kabara (1984b) found that 500 ppm Lauricidin® had dramatic inhibitory effects with anaerobic bacteria when combined with sorbic acid (3:1 ratio, respectively). Baker et al. (1982) also found that 250 ppm Lauricidin® combined with 0.2% citric acid or 0.2% ascorbic acid had a more prounounced effect upon microbial development and the time required to reach microbial spoilage was extended by 5-8 days depending on the meat product. They concluded that the addition of citric acid or ascorbic acid is essential to obtain significant effects with Lauricidin® to extend shelf-life of meats. Oh and Marshall (1992) reported that the MIC of monolaurin (10 and 3 µg/ml) decreased when the pH was reduced from 7.0 to 5.0, respectively, against *L. monocytogenes*. It seems that monolaurin is more effective when coupled with either a chelating agent, an organic acid or by decreasing the pH of the test medium. Kabara (1993) stated that the preservation systems involving monolaurin

(Lauricidin®) are more effective when combined with a food-grade phenolic and/or chelator than alone. However, this effect was not found in the present study when disodium pyrophosphate, horseradish oil and liquid smoke were combined with Lauricidin®. Perhaps the phenolics and chelators used in the present tests were not adequate and that different food-grade phenolic and/or chelators combined with Lauricidin® would result in more promising inhibition.

Disodium pyrophosphate alone had no effect on *Lc. mesenteroides* ssp. *mesenteroides*, and *Lb. sakei* (Figures 19-22). This is unfortunate since other researchers have found that this phosphate (0.4%) was able to delay development of toxicity by *Clostridium botulinum* in peptone-yeast extract-glucose broth (Wagner and Busta, 1983). However, when disodium pyrophosphate was combined with Lauricidin®, it enhanced the ability of Lauricidin® to inhibit *Lc. mesenteroides* ssp. *mesenteroides* until day 10 at 8°C (Figure 21). Disodium pyrophosphate is a linear chain polyphosphate, and has strong metal chelating properties, which can retard the growth of microorganisms (Hargreaves et al., 1972).

Potassium lactate (3% (v/v)) was found to be ineffective against both *Lc. mesenteroides* ssp. *mesenteroides* and *L. sakei* (Figures 23 and 24) at 8°C over a 14 day testing period, even though it has been found effective in various meat trials. For example, it was found that 2 and 3% potassium lactate reduced psychrotrophic populations in pork sausage chubs up to 35 days of storage at refrigeration temperature (Bradford et al., 1993b). Also it was shown that 2% potassium lactate had inhibitory effects against *L. monocytogenes* after 50 days in pork liver sausage stored at 5°C

(Weaver and Shelef, 1993). Perhaps a higher concentration of potassium lactate (>3%) in the inhibitory factor tests may have resulted in a better inhibitory outcome.

4.2.2. Spiral Gradient Endpoint (SGE) Antimicrobial Susceptibility Test

Many combinations of antimicrobials were tested against *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* using the SGE test (Table 9). The results indicated that 4000 ppm disodium pyrophosphate, 4000 ppm phosphate glass, 5000 ppm Alta® 2341, 5000 ppm Alta Mate®, and 30 000 ppm potassium lactate, individually, along with eugenol (500-1000 ppm) were unable to inhibit successfully either organism at 25°C (Table 24). However, at 8°C, 5000 ppm Alta Mate® and 4000 ppm disodium pyrophosphate plus eugenol (500-1000 ppm) were able to strongly inhibit both target microorganisms (Table 25). On average eugenol alone at 25°C was unable to delay the growth of *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* until levels of 778 and 825 ppm, respectively were reached. However, approximately 534 ppm eugenol was able to substantially inhibit *Lc. mesenteroides* ssp. *mesenteroides* and approximately 753 ppm inhibited *Lb. sakei*, respectively, at 8°C. During the course of the SGE testing, it was found that when more than one antimicrobial was combined, the inhibition increased. At both incubation temperatures, the most successful combinations against the target organisms were found when three or more antimicrobials were used (Tables 24 and 25).

Table 24. Interaction of several antimicrobials to cause reduction in the minimum inhibitory concentration of eugenol against *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* at 25°C.

Alta^{R} 2341 (5000 ppm)	Alta Mate^{R} (5000 ppm)	Phosphate glass (4000 ppm)	Disodium pyrophosphate (4000 ppm)	Potassium lactate (30 000 ppm)	EDTA (500 ppm)	*Lc. mesenteroides* ssp. *mesenteroides*	*L. sakei*	Eugenol MAC[a] Average (ppm)
X[b]			X	X		X		514
X			X	X			X	624
X						X		816
X							X	976
X				X	X	X		1000
X				X	X		X	1000
						X		778
							X	825
	X		X	X		X		636
	X		X	X			X	781
	X		X			X		585
	X		X				X	790
	X					X		621
	X						X	819
				X		X		771
				X			X	895
		X				X		801
		X					X	949
			X			X		775
			X				X	890

[a] minimum activity concentration or MIC (minimum inhibitory concentration), determined with the SGE program.

[b] antimicrobial applied within the MRS medium supplemented with 2.5% NaCl + 100 ppm $NaNO_2$.

Table 25. Interaction of several antimicrobials to cause reduction in the minimum inhibitory concentration of eugenol against *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* at 8°C.

Alta^{R} 2341 (5000 ppm)	Alta Mate^{R} (5000 ppm)	Phosphate glass (4000 ppm)	Disodium pyrophosphate (4000 ppm)	Potassium lactate (30 000 ppm)	EDTA (500 ppm)	*Lc. mesenteroides* ssp. *mesenteroides*	*L. sakei*	Eugenol MAC[a] Average (ppm)
X[b]			X	X		X		453
X			X	X			X	490
X						X		532
X							X	825
X				X	X	X		1000
X				X	X		X	1000
						X		534
							X	753
	X		X	X		X		162
	X		X	X			X	329
	X		X			X		190
	X		X				X	335
	X					X		307
	X						X	377
				X		X		693
				X			X	705
		X				X		443
		X					X	836
			X			X		692
			X				X	716

[a] minimum activity concentration or MIC (minimum inhibitory concentration), determined with the SGE program.

[b] antimicrobial applied within the MRS medium supplemented with 2.5% NaCl + 100 ppm $NaNO_2$.

In this series of experiments, it was found that when eugenol was used in a gradient from 500-1000 ppm in MRS agar, 4000 ppm phosphate glass and 5000 ppm Alta® 2341 were less effective than 4000 ppm disodium pyrophosphate and 5000 ppm Alta Mate®. When 500 ppm EDTA was combined with 5000 ppm Alta® 2341 and 30 000 ppm potassium lactate, in the same eugenol gradient, no inhibitory effect was observed against *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* at either incubation temperature (Tables 24 and 25).

It was difficult to assess the inhibitory effect of Lauricidin® on *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei*, because of problems associated with applying Lauricidin® as a gradient onto MRS medium. Lauricidin® crystallised (thick white viscous liquid) either in the stylus of the Autoplater® 4000 or within 5 minutes when applied as a gradient on the medium. Therefore, questions arose as to whether proper concentrations were deposited evenly or whether Lauricidin® was able to diffuse into the MRS medium. It was concluded that a target range of 100-5500 ppm Lauricidin® could not be used with the Autoplater® 4000 under these conditions, and that another type of test(s) would have to be used to determine the effectiveness of Lauricidin® against both organisms. It is interesting to point out that Razavi-Rohani and Griffiths (1994) did not describe any problems with monolaurin in their experiments using the spiral-plater. Two reasons may explain this: 1) they used ethanol (unspecified amount) to dissolve monolaurin compared to propylene glycol (1% in total volume) which was used for this work, suggesting that perhaps propylene glycol affected monolaurin solubility; and 2) the stock concentration of monolaurin used in Razavi-Rohani and Griffiths experiments may not have been as high as used in this work.

It should be noted that after each incubation period, the plates were examined for eugenol odour. Each plate was opened and smelled quickly to determine if any traces of eugenol could be detected. No obvious odours were detected in Petri dishes containing spiral-plated eugenol with an agar overlay. Therefore, it was assumed that the eugenol did not volatilise into the air and cause alterations in the desired test concentrations.

Some of the data generated through these experiments were used to choose combinations of inhibitors that had the most potential for suppressing the growth of both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* in meat trials. The two most promising combinations of agents were 5000 ppm (w/v) Alta Mate®, 400 ppm (v/v) eugenol, 4000 ppm (w/v) disodium pyrophosphate; and 5000 ppm (w/v) Alta Mate®, 30 000 ppm (v/v) potassium lactate, 350 ppm (v/v) eugenol, 4000 ppm (w/v) disodium pyrophosphate.

4.2.3. Agar and Antimicrobial Diffusion Tests

4.2.3.1. Agar Spot Test

The agar spot tests (Table 10) yielded no inhibition of either *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* by the antimicrobials tested. Several attempts were made to decrease the concentration of agar in the medium, from 1.5% to 0.5%, in order to allow the molecules of the antimicrobial to diffuse through the matrix of the medium more easily. If the matrix of the medium is 'tight', this may prevent any inhibition from occurring, because the molecules of the antimicrobial are unable to reach and delay the growth of the organism.

Since eugenol is somewhat volatile, 1.5% agar was mixed with this agent before its application to the pre-poured agar surface in order to minimise this effect. However, this procedure still did not improve inhibition of target organisms. It was difficult at times to determine if any real inhibition had occurred, because the agar-antimicrobial concentrations left a spot where the antimicrobial was applied and the spots were of the same size throughout the test concentration range. Therefore, this indicated that it was probably due to the agar hardening after being applied to the inoculated surface.

The problems associated with some of the antimicrobial agents can be found in the inhibitory factor study summary (section 4.2.1.5.) or below. In this study, EDTA was ineffective against both target organisms. Perhaps this was because its antimicrobial activity was not as effective against Gram-positive bacteria compared to Gram-negative bacteria. The primary role of EDTA in this application was to combine with the cation bridge(s) that hold the lipopolysaccharide to the peptidoglycan layer in Gram-negative bacteria. Once the metal bridge is removed, the lipopolysaccharide layer is stripped off making Gram-negative bacteria more vulnerable to peptidoglycan disrupting agents. Since Gram-positive bacteria do not possess this outer membrane layer, the potential role of EDTA as an antimicrobial may not be important.

Since EDTA is a chelator, it was used in these experiments as a substitute for disodium pyrophosphate. Gill (2000) reported that EDTA was able to inhibit both Gram-positive and negative organisms tested in his experiments with microtitre plates. Therefore, it was believed that EDTA might be more inhibitory towards the target organisms than disodium pyrophosphate.

Sodium tripolyphosphate was also unable to inhibit the growth of both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei*. According to the Merek Index (1976), it is used: in water softening, as a peptising agent, as an emulsifier and dispersing agent, and to preserve meat. However, other literature states that sodium tripolyphosphate has antimicrobial activity which is due to its ability to chelate cations essential for bacteria to grow (Shelef and Seiter, 1993). With this in mind, sodium tripolyphosphate was tested as a replacement for disodium pyrophosphate.

4.2.3.2. Well Diffusion Test

The well diffusion test (Table 11) using allyl isothiocyanate (AIT) was not successful in determining the MICs for *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei*. There were no inhibitory zones created from different concentrations of AIT in the wells at both 8°C and 25°C. Therefore, it was concluded that AIT, at the 500-1000 ppm range, was not sufficient to inhibit both target organisms at these incubation temperatures. Possible explanations for AIT failing to inhibit the target organisms were presented in the inhibitory factor study summary (section 4.2.1.5.).

Another experiment was conducted where the target microorganisms were incorporated directly into the AIT test solutions before being placed into wells. During the course of the incubation period, each well became just as cloudy as the control. This indicated that both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* were able to grow even at the highest concentration of AIT (1000 ppm) applied within the well.

4.2.3.3. Disc Assay Test

The disc assay tests (Table 12) failed to provide any help to determine which antimicrobial(s) alone or in combinations would delay the growth of both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei*. It was unfortunate that the disc assay tests did not provide any useful information since this test was carried out to confirm some of the findings found in the SGE tests.

Several alterations to the tests were performed with no success. For example, when eugenol was applied to the disc, a drop of molten agar was applied as well. This helped to prevent the volatile eugenol from escaping into the air causing a lower concentration on the paper disc. Another attempt made was to combine the molten agar with eugenol before application onto the paper disc. The last alternative was to dip the disc into the concentrated eugenol solution before placing the paper disc onto the medium, followed by an overlay of agar. None of these changes proved to be successful in attempting to determine the MIC of test antimicrobials.

Several additional problems arose during the disc assay tests. One such problem occurred when several discs tended to lift up and move when the agar was applied as an overlay. This caused results to be inaccurate, because once moved, the disc loses initial contact with the agar and the diffusion process is interrupted (Acar and Goldstein, 1986). Therefore, to solve this problem, a drop of agar was placed on top of or slightly off to one side of the disc after the antimicrobial was applied. This additional drop of agar helped the disc to adhere to the medium. However, another problem resulted from this technique. There were consistent zones of approximately the same size around each disc caused by the drop of agar. This happened even when the antimicrobial concentrations

had changed from disc to disc. Therefore, this method was not helpful in determining the MIC of antimicrobials tested.

It was disappointing that Alta® 2341 and Alta Mate® were unable to delay the growth of both target organisms. There had been some success with Alta® 2341 in extending shelf-life of cooked chicken and turkey breasts (Rozum and Maurer, 1997; Schlyter et al., 1993, respectively). Rozum and Maurer (1997) reported that 0.75% and 1% Alta® 2341 provided greater inhibition of aerobic bacteria compared to lower concentrations (0.25 and 0.5%) or the control (untreated) in chicken breasts held at refrigerated temperatures, even after 5 weeks of storage. Schlyter et al. (1993) found that 0.5-0.75% Alta® 2341 combined with 0.5% diacetate in turkey breast slurries had an anti-listerial effect after 7 days at 25°C. However, in this study, Alta® 2341 failed to inhibit a cocktail of *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* in both bologna and ham sausage samples. Since Alta Mate® is a bacteriocin product, similar to Alta® 2341, expectations were that it should have showed some success.

Other possible explanations for the failure of the antimicrobials tested can be found in inhibitory factor study summary (section 4.2.1.5.) and agar spot test discussion (section 4.2.3.1.).

4.2.3.4. Summary

There are other possible reasons why the agar spot, well diffusion and disc assay tests were unsuccessful. Zaika (1988) pointed out that the effect of antimicrobial activity of spices against microorganisms depends on a number of issues. For instance, she suggested that the sensitivity to a particular antimicrobial agent might be dependent upon

the strain of microorganisms used. This is true since Gram-positive strains are more sensitive than Gram-negative strains, and LAB are the most resistant of all Gram-positive bacteria. During a previous experiment involving meat fermentation, she noted that there were increased amounts of acid produced by LAB in the presence of spices, and that the effect increased with increasing spice concentrations (Zaika et al. 1978). She later discovered that the stimulatory factor of spices found in solvent-insoluble, acid-soluble fractions was manganese, which is an element that is essential to LAB for growth and subsequently production of lactic acid (Zaika and Kissinger, 1984). Therefore, some spices can serve as substrates for microbial growth and/or biochemical activities of microorganisms instead of inhibiting them. For the well diffusion and agar spot tests, a chelator such as EDTA should have been incorporated with the antimicrobial agent(s) to chelate ions used for growth by the bacteria tested. However, when chelators were mixed with antimicrobial agents in the disc assay tests the combinations still failed to inhibit both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* at 25°C and 8°C for 48 h and 7 days, respectively. Barry (1986) has mentioned some problems associated with the disc assay test, which are described below. Zaika (1988) has also pointed out that during incubation, loss of the inhibitory substance through evaporation may occur; therefore, allowing growth to take place. This may have happened to some extent when AIT was tested using the well diffusion test and eugenol with the agar spot test, but agar overlays were used to reduce volatilisation.

A problem that can occur when using disc assay tests is that the inhibitory effect of the essential oil can be dependent on its ability to diffuse through the agar medium (Zaika, 1988; Kabara, 1984; Davidson and Parish, 1989). It has been suggested that the

disc assay test should not be used alone in screening for antimicrobial activity since it provides false information for lipophilic oils, such as Lauricidin®. The inhibitory activity is limited by the ability of the antimicrobial to move from the disc and subsequently delay growth of the target organisms (Kabara, 1984).

In an interesting proposal made by Barry (1986), it was suggested that when the antimicrobial agent is applied to the disc, the concentration of the antimicrobial should be 50 times the volume capacity of the disc. Meaning that when a known volume of an antimicrobial agent is delivered to each disk, resulting disk potency is a function of the retained volume as well as concentration of the antimicrobial agent used to load the disk. Thus, if normal disc capacity were 20μl, the starting concentration of the antimicrobial agent should be at least 1000 ppm. This could have been taken into account, but the upper limit of antimicrobial concentrations was often limited by sensory considerations. Barry (1986) also pointed out that the concentration of both the agar and the various ions within the medium should be taken into account, and suggested testing the antimicrobials on several different types of media to determine whether differences in zones of inhibition occur.

In all three tests, the MRS medium may have played a role in preventing the antimicrobial agents from affecting the target organisms. The MRS medium contains cations, such as magnesium, which are free to ionise and may not have been fully bound by added polyphosphates or other complexes. These free cations within the medium can affect the activity of the antimicrobial agent (Barry, 1986).

4.2.4. Growth Inhibition Test

The growth inhibition test was carried out using antimicrobials alone or in combination against both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* (Table 13). During each set of tests, both organisms were spiral-plated to determine their $CFU \cdot ml^{-1}$ within 48 h at 25°C. In all cases, both target organisms grew to 10^8 $CFU \cdot ml^{-1}$, indicating that both organisms were viable (data not presented).

Hall and Maurer (1986) found that propylene glycol inhibited *C. botulinum* at a concentration of 14.3% or greater. Propylene glycol was therefore tested to see if it was inhibitory against *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* since propylene glycol was used to dissolve Lauricidin® in this work. The results indicated that over a range of 50-1000 ppm, propylene glycol had no effect on the growth of both target organisms at 8°C and 25°C, because there was visual growth seen in each well after 48 h at 8°C and after 12 h at 25°C (data not presented). Growth continued despite increased concentrations of propylene glycol. These results are in agreement with those of Wang and Johnson (1997) where 0.1% propylene glycol was not inhibitory against *L. monocytogenes*. They also found that when 0.1 to 0.2% propylene glycol was combined with 50 µg/g monolaurin, the combination had an inhibitory effect on *L. monocytogenes* at 37°C for 6 days.

In our tests, Lauricidin® alone allowed an increase in growth until the 4^{th} day (96 h) of incubation at 25°C, which was followed by a steady decrease in absorbance values of the test media by both target organisms (data not presented). At 8°C, there was visual growth of both organisms after the 5^{th} day of incubation (data not presented). Kabara (1984) stated that Lauricidin® generally is more active at higher rather than lower

temperatures (exact temperatures not specified), however, this was not seen in this experimental trial. This may indicate that the test did not work properly. However, Wang and Johnson (1992) found that 10-20µg/ml monolaurin inactivated *L. monocytogenes* in skim milk at 4°C, but was less inhibitory at 23°C. The inability for Lauricidin® to delay the growth of both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* in the present study is also comparative with results obtained by Oh and Marshall (1992). They also found that 1% stock solution of monolaurin was unable to inhibit four strains of *L. monocytogenes* at 35°C for 24 h. However, they did discover that by lowering the pH of the test broth from pH 7.0 to 5.0, monolaurin showed inhibitory effects against the target strains. They reconfirmed this data in a report (Oh and Marshall, 1993) where 5-9 µg/ml monolaurin in tryptic soy broth with yeast extract, adjusted to pH 5.0, 5.5, or 7.0, was inoculated with *L. monocytogenes* and found increased inhibitory effects with a decrease in pH. Perhaps lowering the pH of the broth in the present study may have helped to delay the growth of *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei*.

Lauricidin® combined with Alta Mate® at 8°C resulted in a continuous increase in absorbance of *Lc. mesenteroides* ssp. *mesenteroides* throughout the test period, whereas, *Lb. sakei* increased in growth to day 5 and then began to decrease in absorbance values as the test period continued (data not presented). However, visual growth at 8°C was seen only at the last day of testing (day 7) (data not presented). At 25°C, there was visual growth seen in all wells after 24 h, and after 48 h a white pellet (or thick white viscous liquid) was also clearly seen at the bottom of each well. These white pellets were seen only in uninoculated plates of Lauricidin® plus Alta Mate® after 48 h of incubation

at 25°C. These white pellets were not similar to the growth found previously in inoculated plates. It was suggested that perhaps Lauricidin®, in the presence of Alta Mate®, precipitated out of the broth solution.

Lauricidin® plus disodium pyrophosphate at 8°C yielded an increase in absorbance values of both target organisms until the 4th day, which was followed by a decrease in absorbance values. At 8°C, there was no visual growth or cloudiness seen throughout the test period indicating that perhaps the growth was small and not as turbid compared to other combinations, and that it was probably due to the disodium pyrophosphate (data not presented). This combination was unable to prevent the growth of both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* at 25°C. At 25°C, there was visible growth after 12 h of incubation even though there was a steady decrease in absorbance values of both organisms (data not presented). This visual growth was probably a mixture of viable stationary phase and dead cells, making the broth within the well appear to be cloudy. The failure of Lauricidin® combined with disodium pyrophosphate (a chelator) is discouraging since Razavi-Rohani and Griffiths (1994), and Blaszyk and Holley (1998) both found that Lauricidin® combined with chelators was able to inhibit a range of microorganisms at 25 and 37°C, and 7 and 18°C, respectively.

The combination of Lauricidin® and potassium lactate resulted in a decrease in absorbance values for both organisms at 25°C (data not presented). At 8°C, there were continuous increases in absorbance values of both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* during the test period, indicating that this combination did not delay the growth of both organisms (data not presented).

The combination of Lauricidin® plus Alta Mate® plus disodium pyrophosphate resulted in increased absorbance values of both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* at 8°C (data not presented). This was not seen at 25°C, where there was a continuous decline in absorbance values of both target organisms (data not presented). However, there was visible growth within 24 h of inoculation by both target organisms at 8°C and 25°C. Again, there were white pellets (or thick white viscous liquid) also visible at 25°C only, suggesting that at this temperature, Lauricidin® and Alta Mate® probably precipitated. This happened in the presence and absence of disodium pyrophosphate at 25°C. There appears to be an interaction between Lauricidin® and Alta Mate® that is worth investigating.

Data from the combination of Lauricidin® plus Alta Mate® plus disodium pyrophosphate plus potassium lactate were similar to the above results. Both organisms yielded increased absorbance values at 8°C, but at 25°C the absorbance values decreased (data not presented). There was visible growth within 24 h of inoculation at both incubation temperatures. Again, white pellets (or thick white viscous liquid) were found at 25°C only.

In summary, it seems that Lauricidin® alone or in combination was not successful in delaying the growth of either *Lc. mesenteroides* ssp. *mesenteroides* or *Lb. sakei* at 8°C. Also, Lauricidin® and Alta Mate® may not be useful at 25°C, since they interacted to form a white insoluble material at the bottom of the microtitre plates. This indicated that perhaps either or both antimicrobials precipitated out of solution, or that there was a chemical reaction taking place when the two antimicrobials were combined.

A study was carried out, at both incubation temperatures, to determine what concentration of Lauricidin® (50-1000 ppm) caused white pellets to form in the presence of 0.5% Alta Mate®. At 8°C, results indicated that crystal-like shapes (similar to snow flake appearance) were formed at 250 ppm Lauricidin® or higher but not at 50, 100, 150 and 200 ppm (data not presented). At 25°C, no crystal-like shapes or pellets were formed at all (data not presented). It can be concluded that at 8°C, Lauricidin® precipitates out of the medium at ≥250 ppm and may affect its ability to inhibit the target organisms. Therefore, 200 ppm Lauricidin® or less should be used under these conditions. At 25°C, it was found that Lauricidin® up to 1000 ppm did not precipitate, but when this antimicrobial was combined with 0.5% Alta Mate®, precipitation occurred.

4.3. Cooked Cured Meat Tests

4.3.1. Visual and Odour Analyses

4.3.1.1. Visual Analysis

Visual characteristics were noted of untreated and treated bologna and ham coins from all treatments. Untreated and treated bologna coins were pink in colour, and the emulsion was very homogenous. Untreated ham coins appeared to be light pink in colour with chunks of meat slightly darker reddish colour, which was typical of commercial products. Treated ham coins were also light pink in colour, but there were no visible chunks of meat present. The treated ham emulsion was very homogenous in colour, similar to untreated and treated bologna emulsions. This was probably due to the ham emulsion being chopped and mixed with the antimicrobials prior to being stuffed into casings, although there may have been some loss in colour of meat pieces which had been

reduced in size. All treated ham samples had the same appearance, therefore, it was difficult to determine whether an antimicrobial or the extra mechanical treatment had changed the treated ham emulsions.

When both bologna and ham coins were vacuum-packaged in low-O_2 permeable bags, there was very little liquid surrounding the coins. This was taken to indicate that the protein binding ability of the meat tissue was largely unaffected by the chemical and thermal treatments used to prepare samples for inoculation.

4.3.1.2. Odour Analysis

Odour was examined immediately after each bag was opened for all bologna and ham treatments throughout the testing period. Appendices 5-8 display the results for all untreated, treated, uninoculated and inoculated samples. A sour odour, loss of vacuum, or a combination of both tended to become noticeable around the 3rd week of storage. This usually happened with inoculated untreated and -treated bologna and ham coins. The development of off odour was due to growth of inoculated microflora in these treatments. Uninoculated untreated and -treated bologna and ham coins tended to be free from undesirable odour and loss of vacuum until the last week of testing. There was one exception to this observation. The treatment consisting of 500 ppm (w/w) Alta Mate® plus 350 ppm (w/w) eugenol plus 0.4% (w/w) disodium pyrophosphate plus 3% (w/w) potassium lactate at week 5 had lost its vacuum. This was probably due to the application of a poor heat seal at vacuum-packaging since this problem did not occur with parallel samples analysed the following week.

4.3.2. Colour Determination

Results from combinations consisting of 0.5% (w/w) Alta Mate® plus 350 or 400 ppm (w/w) eugenol plus 0.4% (w/w) disodium pyrophosphate plus 3% (w/w) potassium lactate are presented in Tables 26-29. Results from combinations consisting of 1% (w/w) Alta Mate® plus 1000 ppm (w/w) eugenol plus 0.4% (w/w) disodium pyrophosphate with and without 3% (w/w) potassium lactate are found in Tables 30-33.

Uninoculated untreated bologna was significantly lighter (increased "L" values) than uninoculated bologna treated with 0.5% (w/w) Alta Mate® plus 400 ppm (w/w) eugenol plus 0.4% (w/w) disodium pyrophosphate (Table 26). However, this was not found in inoculated untreated and treated bologna samples. Uninoculated ham samples treated with 0.5% (w/w) Alta Mate® plus 400 ppm (w/w) eugenol plus 0.4% (w/w) disodium pyrophosphate (Table 27) had significantly increased "L" values and decreased "a" (redness) values compared with uninoculated untreated ham. Also, it was found that there were significant increases in "L" values for inoculated treated ham samples compared to samples that were inoculated and untreated.

Uninoculated ham samples treated with 0.5% (w/w) Alta Mate® plus 350 ppm (w/w) eugenol plus 0.4% (w/w) disodium pyrophosphate plus 3% (w/w) potassium lactate were lighter and more bleached (decreased "a" values) compared to uninoculated untreated ham samples (Table 28). During the same testing period, inoculated treated ham samples were also significantly lighter in colour than the untreated inoculated ham samples. However, uninoculated and inoculated, untreated and treated bologna samples of the same combination were not found to be significantly different in all three hunterlab measurements (Table 29).

As antimicrobial agents increased, it was found that untreated uninoculated and inoculated bologna samples were significantly increased in "b" (yellowness) values compared to uninoculated and inoculated bologna treated with 1% (w/w) Alta Mate® plus 1000 ppm (w/w) eugenol plus 0.4% (w/w) disodium pyrophosphate (Table 30). It was also interesting to find that treated uninoculated and inoculated bologna samples had significantly higher "a" (redness) values compared to untreated uninoculated and inoculated samples. For ham treated with 1% (w/w) Alta Mate® plus 1000 ppm (w/w) eugenol, the uninoculated and inoculated samples were significantly lighter and were more red in colour compared to untreated uninoculated and inoculated ham samples (Table 31).

Uninoculated bologna formulated with 1% (w/w) Alta Mate® plus 1000 ppm (w/w) eugenol plus 0.4% (w/w) disodium pyrophosphate plus 3% (w/w) potassium lactate had significant increased "a" values, and significant decreased "L" and "b" values compared to untreated uninoculated bologna samples (Table 32). Inoculated untreated bologna samples were only significantly different in "b" values when compared to inoculated treated bologna. It was interesting to find that uninoculated and inoculated ham formulated with 1% (w/w) Alta Mate® plus 1000 ppm (w/w) eugenol plus 3% (w/w) potassium lactate had significantly increased "L" and "a" values compared to untreated uninoculated and inoculated ham samples (Table 33). As well, inoculated untreated ham was significantly different for "b" values compared to inoculated treated ham.

Given previous results it was surprising that there were no significant differences in any hunterlab values when uninoculated and inoculated bologna treated with 1% (w/w)

Alta Mate® plus 1000 ppm (w/w) eugenol plus 0.4% (w/w) disodium pyrophosphate with or without 3% (w/w) potassium lactate were compared (Table 34). It was also noted for uninoculated ham treated with 1% (w/w) Alta Mate® plus 1000 ppm (w/w) eugenol, the samples were slightly darker and more red in colour with the addition of 3% (w/w) potassium lactate compared to uninoculated ham samples without potassium lactate (Table 35). The same was true for inoculated ham treatments, except that the ham was significantly darker with the addition of 3% potassium lactate. The darkening effect seen in ham samples was due to potassium lactate. The inclusion of lactate in ham treatments reduced the extent of colour lightening caused by Alta Mate® and eugenol (Table 31). Lamkey et al. (1991) found that sodium lactate did not affect the lean colour of retail fresh pork sausage patties displayed in a retail case at 2 ± 3°C for up to 45 days, but resulted in more rapid surface discolouration. Pigment instability may have been, in part, due to oxidative reactions induced during the extra chopping step given all treated ham samples. As mentioned previously, ham samples were first frozen at -20°C before the meat trials began, which may have had an impact on the colour of the samples since cured meat products are susceptible to oxidative and textural changes under frozen conditions (Cassens, 1994).

All colour results mentioned differ from the hunterlab values obtained by Bradford et al. (1993a) where low-fat fresh pork sausages stored at 5-7°C for 12 d were not affected by the addition of 2% potassium lactate. Bradford et al. (1993b) also found that 2% potassium lactate did not alter the colour of low-fat fresh pork chubs stored at 5-7°C for up to 35 days. However, the authors did find that the addition of 3% potassium lactate had an affect on "L" and "a" values for the meat products. The present

Table 26. Colour stability[a] of bologna formulated with 0.5% (w/w) Alta Mate[R] + 400 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate during storage at 8°C under vacuum.

	Untreated[b] uninoculated					Untreated inoculated[c]			
Week	0	1	3	5	9	0	1	3	5
L	51.2[de]	52.3[e]	53.2[e]	52.7[e]	52.4[e]	51.1	52.2	53.5	53.3
a	8.5	8.1	8.5	8.3	8.9	8.6	8.7	8.9	8.8
b	10.2	10.2	10.2	10.1	10.2	10.2	10.1	10.1	10.2
	Treated uninoculated					Treated inoculated			
Week	0	1	3	5	9	0	1	3	5
L	50.3[d]	50.1[d]	51.2[d]	49.8[d]	50.1[d]	50.3	50.7	51.3	52.2
a	7.8	8.4	8.7	8.7	8.9	7.9	8.7	8.9	8.9
b	10.6	10.3	10.2	10.2	10.5	10.6	10.2	9.9	10.1

[a] measured by a Miniscan spectrophotometer.

[b] commercial formulation with no additions.

[c] inoculated with an equal mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* cells to yield 3 log CFU.cm^{-2}.

[d] n=4.

[e-g] values followed by a different superscript are significantly different (alpha = 0.05).

Table 27. Colour stability[a] of ham formulated with 0.5% (w/w) Alta Mate[R] + 400 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate during storage at 8°C under vacuum.

	Untreated[b] uninoculated					Untreated inoculated[c]			
Week	0	1	3	5	9	0	1	3	5
L	48.2[oe]	49.9[e]	51.3[e]	50.4[e]	50.4[e]	47.8[l]	50.1[l]	50.1[l]	51.2[l]
a	6.4[n]	6.9[n]	7.4[n]	7.6[n]	7.1[n]	6.4	7.4	8.2	8.1
b	7.9	7.2	6.9	7.3	7.2	7.9	7.0	6.9	7.0
	Treated uninoculated					Treated inoculated			
Week	0	1	3	5	9	0	1	3	5
L	53.6[g]	53.6[g]	52.4[g]	53.4[g]	53.4[g]	53.6[m]	54.2[m]	54.3[m]	54.6[m]
a	4.7[k]	5.4[k]	6.2[k]	6.0[k]	6.2[k]	4.7	6.6	7.1	7.7
b	8.1	7.7	6.9	7.3	7.5	8.0	7.2	7.0	7.3

[a] measured by a Miniscan spectrophotometer.

[b] commercial formulation with no additions.

[c] inoculated with an equal mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* cells to yield 3 log CFU.cm^{-2}.

[d] n=4.

[e, g, h, k, l, m] values followed by a different superscript are significantly different (alpha = 0.05).

Table 28. Colour stability[a] of ham formulated with 0.5% (w/w) Alta Mate[R] + 350 ppm (w/w) eugenol + 3% (w/w) potassium lactate + 0.4% (w/w) disodium pyrophosphate during storage at 8°C under vacuum.

	Untreated[b] uninoculated					Untreated inoculated[c]			
Week	0	1	3	5	9	0	1	3	5
L	48.5[de]	49.6[e]	50.0[e]	48.6[e]	51.1[e]	48.5[l]	50.0[l]	50.8[l]	49.8[l]
a	6.7[n]	6.8[n]	6.9[n]	7.8[n]	7.2[n]	6.8	7.0	7.8	8.1
b	7.9	7.2	6.6	6.6	7.0	7.5	7.1	6.6	6.9
	Treated uninoculated					Treated inoculated			
Week	0	1	3	5	9	0	1	3	5
L	54.7[g]	54.6[g]	54.5[g]	54.2[g]	54.3[g]	54.9[m]	54.8[m]	55.7[m]	55.9[m]
a	5.1[k]	5.3[k]	5.7[k]	5.3[k]	5.7[k]	5.1	5.8	7.0	6.9
b	8.0	7.7	6.9	7.4	7.3	8.0	7.5	7.0	7.0

[a] measured by a Miniscan spectrophotometer.

[b] commercial formulation with no additions.

[c] inoculated with an equal mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* cells to yield 3 log CFU.cm^{-2}.

[d] n=4.

[e, g, h, k, l, m] values followd by a different superscript are significantly different (alpha = 0.05).

Table 29. Colour stability[a] of bologna formulated with 0.5% (w/w) Alta Mate[R] + 350 ppm (w/w) eugenol + 3% (w/w) potassium lactate + 0.4% (w/w) disodium pyrophosphate during storage at 8°C under vacuum.

	Untreated[b] uninoculated					Untreated inoculated[c]			
Week	0	1	3	5	9	0	1	3	5
L	51.0[d]	53.4	52.9	53.2	53.1	51.9	53.3	53.2	53.6
a	8.4	8.0	8.4	8.4	8.5	8.5	8.4	9.1	8.7
b	10.2	10.5	10.0	10.2	10.6	10.7	10.2	10.0	10.1
	Treated uninoculated					Treated inoculated			
Week	0	1	3	5	9	0	1	3	5
L	50.8	52.7	52.6	53.2	52.2	52.0	52.7	53.2	53.2
a	7.8	8.3	8.3	8.2	7.8	8.2	8.2	8.4	8.5
b	10.5	10.3	9.8	9.9	10.4	10.2	10.1	9.8	9.9

[a] measured by a Miniscan spectrophotometer.

[b] commercial formulation with no additions.

[c] inoculated with an equal mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* cells to yield 3 log CFU.cm^{-2}.

[d] n=4. There were no significant differences among values (alpha = 0.05).

Table 30. Colour stability[a] of bologna formulated with 1% (w/w) Alta Mate[R] + 1000 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate during storage at 8°C under vacuum.

				Untreated[b] uninoculated				
Week	0	1	2	3	4	5	6	7
L	53.2[c]	50.9	51.3	51.1	51.1	50.3	51.1	52.2
a	9.2[e]	9.0[e]	9.0[e]	8.9[e]	9.1[e]	9.3[e]	8.7[e]	8.9[e]
b	10.3[n]	10.0[n]	10.2[n]	10.1[n]	10.1[n]	10.0[n]	10.5[n]	10.2[n]
				Treated uninoculated				
Week	0	1	2	3	4	5	6	7
L	53.4	51.2	50.9	50.8	50.8	50.3	50.9	51.7
a	9.4[g]	9.2[g]	9.4[g]	9.3[g]	9.4[g]	9.2[g]	9.2[g]	9.1[g]
b	9.5[k]	9.8[k]	9.8[k]	9.9[k]	9.9[k]	9.9[k]	9.9[k]	9.9[k]
				Untreated inoculated[d]				
Week	0	1	2	3	4	5	6	7
L	51.3	51.8	49.5	51.9	52.6	49.5	50.3	52.8
a	8.2[i]	9.0[i]	9.1[i]	8.9[i]	9.0[i]	9.2[i]	8.8[i]	8.7[i]
b	9.8[n]	10.0[n]	9.9[n]	10.0[n]	9.9[n]	9.8[n]	9.7[n]	9.8[n]
				Treated inoculated				
Week	0	1	2	3	4	5	6	7
L	53.4	51.5	51.1	51.6	51.9	51.3	51.8	52.4
a	9.5[m]	9.2[m]	9.6[m]	9.4[m]	9.4[m]	9.5[m]	9.4[m]	9.2[m]
b	9.5[p]	9.6[p]	9.7[p]	9.6[p]	9.4[p]	9.4[p]	9.5[p]	9.3[p]

[a] measured by a Miniscan Spectrophotometer.

[b] commercial formulation with no additions.

[c] n=4.

[d] inoculated with an equal mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* cells to yield 3 log CFU.cm^{-2}.

[e, g, h, k, l, m, n, p] values followed by a different superscript are significantly different (alpha = 0.05).

Table 31. Colour stability[a] of ham formulated with 1% (w/w) Alta Mate[R] + 1000 ppm (w/w) eugenol during storage at 8°C under vacuum.

				Untreated[b] uninoculated				
Week	0	1	2	3	4	5	6	7
L	52.7^{ce}	52.5^{e}	52.9^{e}	52.5^{e}	52.1^{e}	52.4^{e}	52.3^{e}	53.2^{e}
a	5.0^{n}	4.9^{n}	4.8^{n}	5.5^{n}	5.9^{n}	5.3^{n}	5.5^{n}	5.9^{n}
b	7.2	7.5	7.5	6.9	6.7	7.1	6.8	6.5
				Treated uninoculated				
Week	0	1	2	3	4	5	6	7
L	54.2^{g}	53.7^{g}	53.7^{g}	53.3^{g}	53.3^{g}	53.8^{g}	52.9^{g}	53.4^{g}
a	6.5^{k}	6.6^{k}	6.5^{k}	6.5^{k}	6.6^{k}	6.6^{k}	6.3^{k}	6.7^{k}
b	6.7	6.8	6.9	6.8	6.9	7.0	7.2	7.0
				Untreated inoculated[d]				
Week	0	1	2	3	4	5	6	7
L	50.4^{l}	53.0^{l}	53.0^{l}	53.2^{l}	52.9^{l}	53.7^{l}	53.6^{l}	52.0^{l}
a	5.8	6.2	7.1	7.0	7.2	6.9	6.8	7.2
b	6.7^{n}	6.5^{n}	6.1^{n}	5.9^{n}	6.1^{n}	6.1^{n}	6.3^{n}	5.9^{n}
				Treated inoculated				
Week	0	1	2	3	4	5	6	7
L	54.5^{m}	54.3^{m}	54.4^{m}	54.5^{m}	54.7^{m}	55.1^{m}	55.3^{m}	53.7^{m}
a	6.3	6.6	7.1	7.0	7.1	7.2	7.2	7.5
b	7.1^{p}	6.9^{p}	6.9^{p}	6.9^{p}	6.9^{p}	6.9^{p}	6.8^{p}	6.4^{p}

[a] measured by a Miniscan Spectrophotometer.

[b] commercial formulation with no additions.

[c] n=4.

[d] inoculated with an equal mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* cells to yield 3 log CFU.cm^{-2}.

[e, g, h, k, l, m, n, p] values followed by a different superscript are significantly different (alpha = 0.05).

Table 32. Colour stability[a] of bologna formulated with 1% (w/w) Alta Mate[R] + 1000 ppm (w/w) eugenol + 3% (w/w) potassium lactate + 0.4% (w/w) disodium pyrophosphate during storage at 8°C under vacuum.

Untreated[b] uninoculated									
Week	0	1	2	3	4	5	6	7	9
L	51.7[ce]	52.3[e]	51.7[e]	51.6[e]	51.9[e]	51.2[e]	51.9[e]	51.3[e]	50.7[e]
a	8.8[n]	8.6[n]	8.9[n]	8.9[n]	8.8[n]	9.1[n]	9.0[n]	9.1[n]	9.2[n]
b	10.1[i]	10.1[i]	10.2[i]	10.4[i]	10.5[i]	10.3[i]	10.3[i]	10.5[i]	10.3[i]
Treated uninoculated									
Week	0	1	2	3	4	5	6	7	9
L	50.5[g]	50.0[g]	50.4[g]	50.4[g]	50.8[g]	50.8[g]	51.6[g]	50.8[g]	50.9[g]
a	9.4[k]	9.5[k]	9.4[k]	9.4[k]	9.1[k]	9.3[k]	9.0[k]	9.1[k]	9.1[k]
b	9.5[m]	9.7[m]	9.7[m]	9.5[m]	9.8[m]	9.8[m]	9.7[m]	9.8[m]	9.7[m]
Untreated inoculated[d]									
Week	0	1	2	3	4	5	6	7	9
L	51.7	51.3	51.0	52.1	52.1	51.5	52.4	51.8	52.2
a	8.8	9.2	9.3	9.4	8.9	9.2	8.7	9.1	9.2
b	10.1[n]	10.1[n]	10.1[n]	10.1[n]	10.3[n]	9.9[n]	9.9[n]	9.9[n]	9.9[n]
Treated inoculated									
Week	0	1	2	3	4	5	6	7	9
L	50.6	51.2	51.0	51.3	51.3	51.5	52.3	52.1	51.8
a	9.2	9.3	9.4	9.3	9.3	9.4	9.0	9.1	9.2
b	9.3[p]	9.4[p]	9.5[p]	9.6[p]	9.5[p]	9.6[p]	9.3[p]	9.5[p]	9.4[p]

[a] measured by a Miniscan Spectrophotometer.

[b] commercial formulation with no additions.

[c] n=4.

[d] inoculated with an equal mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* cells to yield 3 log CFU.cm^{-2}.

[e, g, h, k, l, m, n, p] values followed by a different superscript are significantly different (alpha = 0.05).

Table 33. Colour stability[a] of ham formulated with 1% (w/w) Alta Mate^{R} + 1000 ppm (w/w) eugenol + 3% (w/w) potassium lactate during storage at 8°C under vacuum.

				Untreated[b] uninoculated					
Week	0	1	2	3	4	5	6	7	9
L	51.2^{ce}	50.5^{e}	50.7^{e}	51.0^{e}	50.9^{e}	49.7^{e}	51.6^{e}	52.6^{e}	51.4^{e}
a	6.1^{n}	5.4^{n}	5.6^{n}	5.7^{n}	5.6^{n}	5.7^{n}	5.8^{n}	7.0^{n}	6.4^{n}
b	6.6	7.0	6.8	6.7	7.1	6.4	6.9	6.3	6.8
				Treated uninoculated					
Week	0	1	2	3	4	5	6	7	9
L	53.5^{g}	53.1^{g}	53.0^{g}	52.9^{g}	52.9^{g}	53.3^{g}	53.1^{g}	53.2^{g}	51.6^{g}
a	6.7^{k}	6.7^{k}	6.8^{k}	7.0^{k}	6.6^{k}	6.7^{k}	6.8^{k}	6.8^{k}	7.4^{k}
b	6.9	6.9	7.1	6.7	7.0	7.0	7.0	7.1	5.9
				Untreated inoculated[d]					
Week	0	1	2	3	4	5	6	7	9
L	51.0^{n}	52.2^{n}	51.8^{n}	53.0^{n}	52.6^{n}	52.9^{n}	52.1^{n}	52.6^{n}	52.4^{n}
a	6.1^{r}	5.4^{r}	6.9^{r}	7.2^{r}	6.8^{r}	6.9^{r}	6.9^{r}	7.3^{r}	6.8^{r}
b	6.5^{t}	6.7^{t}	6.2^{t}	6.1^{t}	5.9^{t}	6.3^{t}	6.3^{t}	6.1^{t}	7.3^{t}
				Treated inoculated					
Week	0	1	2	3	4	5	6	7	9
L	53.3^{p}	52.9^{p}	53.0^{p}	52.8^{p}	52.8^{p}	53.9^{p}	53.3^{p}	53.8^{p}	54.5^{p}
a	6.8^{s}	7.2^{s}	7.3^{s}	7.4^{s}	7.1^{s}	7.7^{s}	7.4^{s}	7.6^{s}	7.4^{s}
b	6.8^{u}	6.7^{u}	6.9^{u}	6.9^{u}	6.7^{u}	7.0^{u}	6.5^{u}	6.9^{u}	7.1^{u}

[a] measured by a Miniscan Spectrophotometer.

[b] commercial formulation with no additions.

[c] n=4.

[d] inoculated with an equal mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* cells to yield 3 log CFU.cm^{-2}.

[e, g, n, k, n, p, r, s, t, u] values followed by a different superscript are significantly different (alpha = 0.05).

Table 34. Colour measurements of cooked bologna formulated with 1% (w/w) Alta Mate^{R} + 1000 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate with and without 3% (w/w) potassium lactate and stored at 8°C.

	Bologna[a]-uninoculated			Bologna[a]-inoculated		
Week	L	a	b	L	a	b
0	53.4	9.4	9.5	53.4	9.5	9.5
1	51.2	9.2	9.8	51.5	9.2	9.6
2	50.9	9.4	9.8	51.1	9.6	9.7
3	50.8	9.3	9.9	51.6	9.4	9.6
4	50.8	9.4	9.9	51.9	9.4	9.4
5	50.3	9.2	9.9	51.3	9.5	9.4
6	50.9	9.2	9.9	51.8	9.4	9.5
7	51.7	9.1	9.9	52.4	9.2	9.3

	Bologna[b]-uninoculated			Bologna[b]-inoculated		
Week	L	a	b	L	a	b
0	50.5	9.4	9.5	50.6	9.2	9.3
1	50.0	9.5	9.7	51.2	9.3	9.4
2	50.4	9.4	9.7	51.0	9.4	9.5
3	50.4	9.4	9.5	51.3	9.3	9.6
4	50.8	9.1	9.8	51.3	9.3	9.5
5	50.8	9.3	9.8	51.5	9.4	9.6
6	51.6	9.0	9.7	52.3	9.0	9.3
7	50.8	9.1	9.8	52.1	9.1	9.5
9	50.9	9.1	9.7	51.8	9.2	9.4

[a] formulated without 3% (w/w) potassium lactate.

[b] formulated with 3% (w/w) potassium lactate.

Table 35. Colour measurements of cooked ham formulated with 1% (w/w) Alta Mate® + 1000 Ppm (w/w) eugenol with and without 3% (w/w) potassium lactate and stored at 8°C.

	Ham[a]-uninoculated			Ham[a]-inoculated		
Week	L	a	b	L	a	b
0	54.2	6.5	6.7	54.5[n]	6.3	7.1
1	53.7	6.6	6.8	54.3[n]	6.6	6.9
2	53.7	6.5	6.9	54.4[n]	7.1	6.9
3	53.3	6.5	6.8	54.5[n]	7.0	6.9
4	53.3	6.6	6.9	54.7[n]	7.1	6.9
5	53.8	6.6	7.0	55.1[n]	7.2	6.9
6	52.9	6.3	7.2	55.3[n]	7.2	6.8
7	53.4	6.7	7.0	53.7[n]	7.5	6.4

	Ham[b]-uninoculated			Ham[b]-inoculated		
Week	L	a	b	L	a	b
0	53.5	6.7	6.9	53.3[k]	6.8	6.8
1	53.1	6.7	6.9	52.9[k]	7.2	6.7
2	53.0	6.8	7.1	53.0[k]	7.3	6.9
3	52.9	7.0	6.7	52.8[k]	7.4	6.9
4	52.9	6.6	7.0	52.8[k]	7.1	6.7
5	53.3	6.7	7.0	53.9[k]	7.7	7.0
6	53.1	6.8	7.0	53.3[k]	7.4	6.5
7	53.2	6.8	7.1	53.8[k]	7.6	6.9
9	51.6	7.4	5.9	54.5[k]	7.4	7.1

[a] formulated without 3% (w/w) potassium lactate.

[b] formulated with 3% (w/w) potassium lactate.

[n, k] values followed by different superscript are significantly different (alpha = 0.05).

inoculated ham results obtained in Table 35 are in agreement with those of Bradford et al. (1993b) where 3% potassium lactate significantly darkened (decreased "L" values) and increased the redness ("a" values) of low-fat fresh pork chubs. The "b" (yellowness) values were unaffected by the presence of potassium lactate in both Bradford et al. (1993a and 1993b) results and in the present study.

4.3.3. Determination of pH and A_w

4.3.3.1. pH

In the combinations tested in the present study, the pH of all uninoculated untreated and treated bologna and ham samples decreased very slowly compared to inoculated untreated and treated bologna and ham samples (appendices 9-16). The pH of the inoculated bologna and ham samples decreased as the shelf-life increased and was correlated with an increase in both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* numbers. Bradford et al. (1993b) also found this to be true with their low-fat fresh pork sausages chubs. Other researchers agree that in general LAB will decrease the pH of meat products as storage progresses (Borch et al., 1996; Korkeala and Björkroth, 1997; von Holy et al., 1991).

The addition of 3% (w/w) potassium lactate helped to delay the decline in pH of both bologna and ham samples formulated with this antimicrobial agent. This is in agreement with results found by Bradford et al. (1993b) where the addition of potassium lactate delayed pH decline of low-fat fresh pork sausage chubs during refrigerated storage. They stated that the delay in pH decline may have been due to the inhibitory effects of potassium lactate on acid producing facultative anaerobes.

4.3.3.2. A_w

Water activity is defined as the ratio of vapour pressure of a solution to that of pure water at a specified temperature. It is important because it affects many food attributes and characteristics. For example, texture, non-enzymatic browning reactions, enzymatic activity, lipid oxidation and microbial growth are influenced by the manipulation of a_w levels (Troller and Scott, 1992).

Both bologna and ham sausages were stored in a -20°C freezer. The sausages were left out at room temperature the previous night before being cut into ~1 cm thick coins. Both bologna and ham coins treated with 0.5% (w/w) Alta Mate® plus 350 or 400 ppm (w/w) eugenol plus 0.4% (w/w) disodium pyrophosphate with and without 3% (w/w) potassium lactate were 20 weeks old. Bologna and ham coins treated with 1% (w/w) Alta Mate® plus 1000 ppm (w/w) eugenol plus 0.4% (w/w) disodium pyrophosphate (bologna only) with and without 3% (w/w) potassium lactate were 18 weeks old. The Decagon a_w machine reported both a_w and the temperature readings of each sample coin.

The untreated bologna had a significantly higher a_w than bologna treated with 0.5% (w/w) Alta Mate® plus 400 ppm (w/w) eugenol plus 0.4% (w/w) disodium pyrophosphate (Table 36). However, untreated and treated ham did not show any significant differences for the same treatment combination (Table 37).

The a_w of untreated ham was significantly higher than that of ham treated with 0.5% (w/w) Alta Mate® plus 350 ppm (w/w) eugenol plus 0.4% (w/w) disodium pyrophosphate plus 3% (w/w) potassium lactate (Table 38). This was also seen in

Table 36. A_w measurements of cooked uninoculated bologna[a] containing 0.5% (w/w) Alta Mate[R] plus 400 ppm (w/w) eugenol plus 0.4% (w/w) disodium pyrophosphate and stored at –20°C.

	Bologna-untreated		Bologna-treated	
Coin	A_w reading	Temperature (°C)	A_w reading	Temperature (°C)
1	0.961[c]	21.6	0.939[d]	22.3
2	0.961[c]	20.7	0.945[d]	21.9
3	0.961[c]	21.3	0.948[d]	21.9
4	0.961[c]	20.7	0.944[d]	22.0
5	0.949[c]	21.0	0.944[d]	22.3
6	NR[b]	NR	0.944[d]	22.3

[a] samples were frozen after preparation, thawed and left overnight at room temperature before measurements were made.
[b] not recorded; temperature reading was not within ± 1.0, therefore not included.
[c] and [d] values are significantly different (alpha = 0.05).

Table 37. A_w measurements of cooked uninoculated ham[a] containing 0.5% (w/w) Alta Mate[R] plus 400 ppm (w/w) plus 0.4% (w/w) disodium pyrophosphate eugenol and stored at –20°C.

	Ham-untreated		Ham-treated	
Coin	A_w reading	Temperature (°C)	A_w reading	Temperature (°C)
1	0.948	21.1	0.972	21.2
2	0.965	21.7	0.967	20.4
3	0.965	20.9	0.967	20.4
4	0.963	21.5	0.972	21.2
5	0.970	21.2	0.966	20.6
6	NR[b]	NR	0.971	20.8

[a] samples were frozen after preparation, thawed and left out overnight at room temperature before measurements were made.
[b] not recorded; temperature reading was not within ± 1.0, therefore not included.

untreated and treated bologna for the same treatment combination (Table 39).

The a_w of uninoculated ham containing 1% (w/w) Alta Mate® plus 1% (w/w) eugenol with and without 3% (w/w) potassium lactate found the a_w of treated ham was significantly lower (Table 40). The a_w of uninoculated bologna prepared with the same combinations also showed significant differences (Table 41). There are no published papers to date that indicate differences in a_w results from use of the above combinations, however, Chen and Shelef (1992) found that 4% potassium lactate reduced the a_w from 0.986 to 0.957 of commercially sterile beef. Weaver and Shelef (1993) also found this to be true, but their results indicated only a slight reduction in a_w when 2 or 3% potassium lactate was added to pork liver sausages.

4.3.4. Microbial Analysis

The combination consisting of 0.5% (w/w) Alta Mate®, 400 ppm (w/w) eugenol and 0.4% (w/w) disodium pyrophosphate (Tables 42-45) was unable to inhibit either *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* in ham or bologna. For both the untreated uninoculated bologna and ham samples, substantial adventitious bacterial growth occurred within three weeks which was earlier than expected. This may have

Table 38. A_w measurements of cooked uninoculated ham[a] containing 0.5% (w/w) Alta Mate[R] + 350 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate + 3% (w/w) potassium lactate and stored at –20°C.

	Ham-untreated		Ham-treated	
Coin	A_w reading	Temperature (°C)	A_w reading	Temperature (°C)
1	0.986[b]	21.7	0.974[c]	22.4
2	0.987[b]	21.5	0.984[c]	22.7
3	0.983[b]	20.8	0.980[c]	21.6
4	0.984[b]	21.0	0.976[c]	21.1
5	0.993[b]	20.9	0.975[c]	22.2
6	0.980[b]	21.3	0.974[c]	22.2

[a] samples were frozen after preparation, thawed and left out overnight at room temperature before measurements were made.
[b] and [c] values are significantly different (alpha = 0.05).

Table 39. A_w measurements of cooked uninoculated bologna[a] containing 0.5% (w/w) Alta Mate[R] + 350 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate + 3% (w/w) potassium lactate and stored at –20°C.

	Bologna-untreated		Bologna-treated	
Coin	A_w reading	Temperature (°C)	A_w reading	Temperature (°C)
1	0.979[b]	21.0	0.977[c]	22.6
2	0.985[b]	20.7	0.972[c]	21.8
3	0.983[b]	21.7	0.980[c]	22.0
4	0.987[b]	20.6	0.983[c]	22.4
5	0.994[b]	20.7	0.985[c]	21.3
6	0.978[b]	20.7	0.977[c]	21.6

[a] samples were frozen after preparation, thawed and left overnight at room temperature before measurements were made.
[b] and [c] values are significantly different (alpha = 0.05).

Table 40. A_w measurements of cooked uninoculated ham[a] containing 1% (w/w) Alta Mate[R] + 1000 ppm (w/w) eugenol with and without 3% (w/w) potassium lactate and stored at –20°C.

	Ham-treated[b]		Ham-treated[c]	
Coin	A_w reading	Temperature (°C)	A_w reading	Temperature (°C)
1	0.977[d]	21.9	0.965[e]	21.8
2	0.982[d]	22.3	0.974[e]	21.6
3	0.975[d]	21.3	0.982[e]	21.5
4	0.985[d]	21.1	0.966[e]	21.4
5	0.978[d]	21.1	0.966[e]	21.7
6	0.971[d]	22.1	0.970[e]	21.7

[a] samples were frozen after preparation, thawed and left overnight at room temperature before measurements were made.
[b] ham without 3% (w/w) potassium lactate.
[c] ham with 3% (w/w) potassium lactate.
[d] and [e] values are significantly different (alpha = 0.05).

Table 41. A_w measurements of cooked uninoculated bologna[a] containing 1% (w/w) Alta Mate[R] + 1000 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate with and without 3% (w/w) potassium lactate and stored at –20°C.

	Bologna-treated[b]		Bologna-treated[c]	
Coln	A_w reading	Temperature (°C)	A_w reading	Temperature (°C)
1	0.973[d]	21.6	0.965[e]	21.3
2	0.986[d]	20.9	0.976[e]	21.6
3	0.971[d]	21.5	0.963[e]	21.1
4	0.982[d]	20.9	0.977[e]	22.0
5	0.972[d]	21.7	0.965[e]	21.3
6	0.980[d]	21.3	0.974[e]	21.2
Average	0.977	21.3	0.970	21.4

[a] samples were frozen after preparation, thawed and left overnight at room temperature before measurements were made.
[b] bologna without 3% (w/w) potassium lactate.
[c] bologna with 3% (w/w) potassium lactate.
[d] and [e] values are significantly different (alpha = 0.05).

Table 42. Growth of bacteria in bologna formulated with 0.5% (w/w) Alta Mate^R plus 400 ppm (w/w) eugenol and 0.4% (w/w) disodium pyrophosphate followed by cooking, inoculation with or without *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* and stored under vacuum at 8°C (untreated control, uninoculated and inoculated).

	Untreated (control)								
	Uninoculated					Inoculated[a]			
Media	weeks of storage					weeks of storage			
	0	1	3	5	9	0	1	3	5
SPC[b]	ND[g]	3.59[h]	6.50	7.42	4.54	2.95	7.05	6.66	7.03
APT[c]	ND	3.49	6.66	7.07	5.26	3.16	7.09	6.79	7.10
M5[d]	ND	3.47	7.07	7.16	5.66	3.02	7.07	6.67	7.70
STAA[e]	ND	3.50	7.06[i]	6.65[i]	5.72	ND	6.87	2.55	6.80
VRBG[f]	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] inoculated by dipping in a mixture of *Lb.sakei* and *Lc. mesenteroides* ssp. *mesenteroides* at 10^4 cells.cm^{-2} prior to packaging.

[b] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[c] all purpose tween agar, spiral plated, anaerobic, 25°C for 2 days.

[d] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[e] streptomycin thallous acetate actidione agar, spiral plated, aerobic, 25°C for 2 days.

[f] violet red bile glucose agar, spiral plated, aerobic, 35°C for 1 day.

[g] none demonstrated.

[h] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[i] results indicate *Brochothrix* was present.

Table 43. Growth of bacteria in bologna formulated with 0.5% (w/w) Alta Mate[R] plus 400 ppm (w/w) eugenol and 0.4% (w/w) disodium pyrophosphate followed by cooking, inoculation with or without *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* and stored under vacuum at 8°C (treated, uninoculated and inoculated).

	Treated[a]								
	Uninoculated					Inoculated[b]			
Media	weeks of storage					weeks of storage			
	0	1	3	5	9	0	1	3	5
SPC[c]	ND[h]	ND	ND	5.19[i]	ND	3.05	7.06	7.09	6.90
APT[d]	ND	ND	ND	5.49	ND	3.18	7.13	6.96	6.99
M5[e]	ND	ND	ND	6.56	ND	3.25	7.18	7.60	8.17
STAA[f]	ND	ND	ND	5.81[j]	ND	3.11	6.83	6.88	6.71
VRBG[g]	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] commercial bolgona formulated with 0.5% (w/w) Alta Mate[R] plus 400 ppm (w/w) eugenol before cooking.

[b] inoculated by dipping in a mixture of *Lb.sakei* and *Lc. mesenteroides* ssp. *mesenteroides* at 10^4 cells.cm^{-2} prior to packaging.

[c] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[d] all purpose tween agar, spiral plated, anaerobic, 25°C for 2 days.

[e] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[f] streptomycin thallous acetate actidione agar, spiral plated, aerobic, 25°C for 2 days.

[g] violet red bile glucose agar, spiral plated, aerobic, 35°C for 1 day.

[h] none demonstrated.

[i] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[j] results indicate *Brochothrix* was present.

Table 44. Growth of bacteria in ham formulated with 0.5% (w/w) Alta Mate[R] plus 400 ppm (w/w) eugenol and 0.4% (w/w) disodium pyrophosphate followed by cooking, inoculation with or without *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* and stored under vacuum at 8°C (untreated control, uninoculated and inoculated).

	Untreated (control)								
	Uninoculated					Inoculated[a]			
Media	weeks of storage					weeks of storage			
	0	1	3	5	9	0	1	3	5
SPC[b]	ND[g]	4.34[h]	4.23	4.14	3.67	3.04	6.70	6.49	6.42
APT[c]	ND	4.11	3.96	4.03	3.50	3.38	6.69	6.46	6.43
M5[d]	ND	4.25	4.02	5.13	3.71	3.30	6.75	6.47	6.42
STAA[e]	ND	4.80	4.66[i]	4.20[i]	ND	3.07	6.63	5.81	5.80
VRBG[f]	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] inoculated by dipping in a mixture of *Lb.sakei* and *Lc. mesenteroides* ssp. *mesenteroides* at 10^4 cells.cm^{-2} prior to packaging.

[b] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[c] all purpose tween agar, spiral plated, anaerobic, 25°C for 2 days.

[d] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[e] streptomycin thallous acetate actidione agar, spiral plated, aerobic, 25°C for 2 days.

[f] violet red bile glucose agar, spiral plated, aerobic, 35°C for 1 day.

[g] none demonstrated.

[h] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[i] results indicate *Brothothrix* was present.

Table 45. Growth of bacteria in ham formulated with 0.5% (w/w) Alta Mate[R] plus 400 ppm (w/w) eugenol and 0.4% (w/w) disodium pyrophosphate followed by cooking, with or without *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* and stored under vacuum at 8°C (treated, uninoculated and inoculated).

	Treated[a]								
	Uninoculated					Inoculated[b]			
Media	weeks of storage					weeks of storage			
	0	1	3	5	9	0	1	3	5
SPC[c]	ND[h]	ND	4.46[i]	ND	ND	3.01	7.14	7.15	7.07
APT[d]	ND	ND	4.00	ND	ND	3.34	7.13	7.10	7.02
M5[e]	ND	ND	3.81	ND	ND	3.11	7.12	7.72	7.28
STAA[f]	ND	ND	ND	ND	ND	3.03	7.07	2.50	6.55
VRBG[g]	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] commercial bolgona formulated with 0.5% (w/w) Alta Mate[R] plus 400 ppm (w/w) eugenol before cooking.

[b] inoculated by dipping in a mixture of *Lb.sakei* and *Lc. mesenteroides* ssp. *mesenteroides* at 10^4 cells.cm^{-2} prior to packaging.

[c] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[d] all purpose tween agar, spiral plated, anaerobic, 25°C for 2 days.

[e] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[f] streptomycin thallous acetate actidione agar, spiral plated, aerobic, 25°C for 2 days.

[g] violet red bile glucose agar, spiral plated, aerobic, 35°C for 1 day.

[h] none demonstrated.

[i] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

been due to the use of a moderately abusive storage temperature or could have been related to the thermal process used for sausage preparation.

The bologna and ham treated with 0.5% (w/w) Alta Mate®, 350 ppm (w/w) eugenol, 0.4% (w/w) disodium pyrophosphate and 3% (w/w) potassium lactate (Tables 46-49) showed similar results. Therefore, it was concluded that neither combination was able to inhibit growth of inoculated *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei*, but they were able to extend the period that treated meats remained free of naturally occurring spoilage bacteria. Treated uninoculated ham and bologna sausages had a shelf-life at 8°C >9 weeks, whereas untreated uninoculated products were usually spoiled by 5 weeks.

It should be noted that the inoculated untreated and treated bologna and ham samples from the two combinations above were spoiled at 5 weeks of storage and microbial analysis was halted. The uninoculated untreated and treated bologna and ham sausages continued to be analysed for microbial growth, because the samples did not spoil until after week 9.

In bologna and ham, the combinations of 1% (w/w) Alta Mate® plus 1000 ppm (w/w) eugenol plus 0.4% (w/w) disodium pyrophosphate (in bologna only) with and without 3% (w/w) potassium lactate were unable to inhibit both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei*. The results for both the treated bologna and ham samples showed a slight decrease in both organisms compared to the controls. However, both combinations (uninoculated treated) were able to effectively inhibit the natural microflora found in vacuum-packaged meat (Tables 50-65), because spoilage did not occur until week 11 and 13. The uninoculated untreated and treated bologna and ham samples were

Table 46. Growth of bacteria in bologna formulated with 0.5% (w/w) Alta Mate[R] + 350 ppm (w/w) eugenol + 3% (w/w) potassium lactate + 0.4% (w/w) disodium pyrophosphate followed by cooking, inoculation with or without *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* and stored under vacuum at 8°C (untreated control, uninoculated and inoculated).

	Untreated (control)								
	Uninoculated					Inoculated[a]			
Media	weeks of storage					weeks of storage			
	0	1	3	5	9	0	1	3	5
SPC[b]	ND[g]	4.09[h]	5.59	7.71	3.84	2.74	6.84	6.98	7.02
APT[c]	ND	3.76	5.31	8.26	4.84	2.98	7.22	7.40	7.06
M5[d]	ND	ND	5.63	8.24	5.09	2.84	7.12	7.71	7.83
STAA[e]	ND	4.11	4.55	5.70[i]	5.17[i]	ND	7.11	5.02	6.93
VRBG[f]	ND	ND	ND	7.64	ND	ND	ND	ND	ND

[a] inoculated by dipping in a mixture of *Lb.sakei* and *Lc. mesenteroides* ssp. *mesenteroides* at 10^4 cells.cm^{-2} prior to packaging.

[b] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[c] all purpose tween agar, spiral plated, anaerobic, 25°C for 2 days.

[d] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[e] streptomycin thallous acetate actidione agar, spiral plated, aerobic, 25°C for 2 days.

[f] violet red bile glucose agar, spiral plated, aerobic, 35°C for 1 day.

[g] none demonstrated.

[h] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[i] results indicate *Brochothrix* was present.

Table 47. *Growth of bacteria in bologna formulated with 0.5% (w/w) Alta Mate[R] + 350 ppm (w/w) eugenol + 3% (w/w) potassium lactate + 0.4% (w/w) disodium pyrophosphate followed by cooking, with or without Lb. sakei and Lc. mesenteroides ssp. mesenteroides and stored under vacuum at 8°C (treated, uninoculated and inoculated).*

	Treated[a]								
	Uninoculated					Inoculated[b]			
Media	weeks of storage					weeks of storage			
	0	1	3	5	9	0	1	3	5
SPC[c]	ND[h]	ND	ND	ND	ND	3.01[i]	6.78	6.65	6.49
APT[d]	ND	ND	ND	ND	ND	2.95	6.85	6.79	6.46
M5[e]	ND	ND	ND	ND	ND	2.99	6.83	7.03	6.70
STAA[f]	ND	ND	ND	ND	ND	ND	6.76	4.93	5.79
VRBG[g]	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] commercial bolgona formulated with 0.5% (w/w) Alta Mate[R] + 350 ppm (w/w) eugenol + 3% (w/w) potassium lactate + 0.4% (w/w) disodium pyrophosphate before cooking.

[b] inoculated by dipping in a mixture of *Lb.sakei* and *Lc. mesenteroides* ssp. *mesenteroides* at 10^4 cells.cm^{-2} prior to packaging.

[c] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[d] all purpose tween agar, spiral plated, anaerobic, 25°C for 2 days.

[e] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[f] streptomycin thallous acetate actidione agar, spiral plated, aerobic, 25°C for 2 days.

[g] violet red bile glucose agar, spiral plated, aerobic, 35°C for 1 day.

[h] none demonstrated.

[i] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

Table 48. Growth of bacteria in ham formulated with 0.5% (w/w) Alta Mate[R] + 350 ppm (w/w) eugenol + 3% (w/w) potassium lactate + 0.4% (w/w) disodium pyrophosphate followed by cooking, inoculation with or without *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* and stored under vacuum at 8°C (untreated control, uninoculated and inoculated).

	Untreated (control)								
	Uninoculated					Inoculated[a]			
Media	weeks of storage					weeks of storage			
	0	1	3	5	9	0	1	3	5
SPC[b]	ND[g]	ND	7.71[h]	8.47	>9.00	3.12	6.52	6.69	6.83
APT[c]	ND	ND	7.42	8.42	>9.00	3.25	6.73	6.63	6.78
M5[d]	ND	ND	7.58	8.75	>9.00	3.09	6.60	7.17	6.73
STAA[e]	ND	ND	2.72	7.68	5.67	ND	6.52	4.86	7.18
VRBG[f]	ND	ND	ND	8.76	ND	ND	ND	ND	ND

[a] inoculated by dipping in a mixture of *Lb.sakei* and *Lc. mesenteroides* ssp. *mesenteroides* at 10^4 cells.cm^{-2} prior to packaging.

[b] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[c] all purpose tween agar, spiral plated, anaerobic, 25°C for 2 days.

[d] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[e] streptomycin thallous acetate actidione agar, spiral plated, aerobic, 25°C for 2 days.

[f] violet red bile glucose agar, spiral plated, aerobic, 35°C for 1 day.

[g] none demonstrated.

[h] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

Table 49. Growth of bacteria in ham formulated with 0.5% (w/w) Alta Mate[R] + 350 ppm (w/w) eugenol + 3% (w/w) potassium lactate + 0.4% (w/w) disodium pyrophosphate followed by cooking, with or without *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* and stored under vacuum at 8°C (treated, uninoculated and inoculated).

	Treated[a]								
	Uninoculated					Inoculated[b]			
Media	weeks of storage					weeks of storage			
	0	1	3	5	9	0	1	3	5
SPC[c]	ND[h]	ND	ND	ND	ND	2.83	6.60	6.99	7.35
APT[d]	ND	ND	3.73[i]	3.73	ND	3.04	7.51	7.31	7.24
M5[e]	ND	ND	ND	ND	ND	3.05	7.40	7.70	7.75
STAA[f]	ND	ND	ND	ND	ND	ND	8.34	7.05[j]	6.81
VRBG[g]	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] commercial ham formulated with 0.5% (w/w) Alta Mate[R] + 350 ppm (w/w) eugenol + 3% (w/w) potassium lactate + 0.4% (w/w) disodium pyrophosphate before cooking.

[b] inoculated by dipping in a mixture of *Lb.sakei* and *Lc. mesenteroides* ssp. *mesenteroides* at 10^4 cells.cm^{-2} prior to packaging.

[c] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[d] all purpose tween agar, spiral plated, anaerobic, 25°C for 2 days.

[e] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[f] streptomycin thallous acetate actidione agar, spiral plated, aerobic, 25°C for 2 days.

[g] violet red bile glucose agar, spiral plated, aerobic, 35°C for 1 day.

[h] none demonstrated.

[i] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[j] results indicate *Brochothrix* was present.

Table 50. Growth of bacteria in bologna formulated with 1% (w/w) Alta Mate^{R} + 1000 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate followed by cooking, and stored under vacuum at 8°C (untreated control, uninoculated).

	Untreated (control) - uninoculated										
Media	weeks of storage										
	0	1	2	3	4	5	6	7	9	11	13
SPC[a]	ND[f,g]	ND	ND	ND	ND	ND	ND	ND	ND	5.46[h]	7.31
APT[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND	5.36[h]	7.26
M5[c]	ND	ND	ND	ND	ND	ND	ND	ND	ND	5.72[h]	6.45
STAA[d]	ND	ND	ND	ND	ND	ND	ND	ND	ND	5.40[h]	ND
VRBG[e]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[h]	ND

[a] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[b] all purpose tween agar, spiral plated, anaerobic, 25°c for 2 days.

[c] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[d] streptomycin thallous acetate actidione agar, spiral plated, aerobic 25°C for 2 days.

[e] violet bile red glucose agar, spiral plated, aerobic, 35°C for 1 day.

[f] none demonstrated.

[g] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[h] n=2, plated in triplicate.

Table 51. Growth of bacteria in bologna formulated with 1% (w/w) Alta Mate^{R} + 1000 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate followed by cooking, inoculation with *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* and stored under vacuum at 8°C (untreated, inoculated).

	Untreated - inoculated[a]								
Media	weeks of storage								
	0	1	2	3	4	5	6	7	9
SPC[b]	3.25[g]	7.00	7.28	6.29	7.07	6.99	6.68	6.16	6.85
APT[c]	2.85	7.17	7.15	7.21	7.03	7.22	7.02	6.88	6.92
M5[d]	2.76	7.24	7.30	7.17	7.10	7.22	7.02	6.92	6.75
STAA[e]	ND[h]	7.27	7.07	6.92	6.91	7.06	6.56	6.52	6.42
VRBG[f]	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] inoculated by dipping in a mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* at 10^4 cells.cm^{-2} prior to packaging.

[b] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[c] all purpose tween agar, spiral plated, anaerobic, 25°C for 2 days.

[d] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[e] streptomycin thallous acetate actidione agar, spiral plated, aerobic, 25°C for 2 days.

[f] violet bile red glucose agar, spiral plated, aerobic, 35°C for 1 day.

[g] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[h] none demonstrated.

Table 52. Growth of bacteria in bologna formulated with 1% (w/w) Alta Mate^{R} + 1000 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate followed by cooking, and stored under vacuum at 8°C (treated control, uninoculated).

	Treated[a] (control) - uninoculated										
Media	weeks of storage										
	0	1	2	3	4	5	6	7	9	11	13
SPC[b]	ND[g,h]	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND
APT[c]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND
M5[d]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND
STAA[e]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND
VRBG[f]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND

[a] commercial bolgona formulated with 1% (w/w) Alta Mate^{R} + 1000 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate before cooking.

[b] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[c] all purpose tween agar, spiral plated, anaerobic, 25°c for 2 days.

[d] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[e] streptomycin thallous acetate actidione agar, spiral plated, aerobic 25°C for 2 days.

[f] violet bile red glucose agar, spiral plated, aerobic, 35°C for 1 day.

[g] none demonstrated.

[h] log_{10} CFU.cm^{-2}, triplicate samples in duplicate.

[i] n=2, plated in triplicate.

Table 53. Growth of bacteria in bologna formulated with 1% (w/w) Alta Mate[R] + 1000 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate followed by cooking, inoculation with *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* and stored under vacuum at 8°C (treated, inoculated).

	Treated[a] - inoculated[b]								
Media	weeks of storage								
	0	1	2	3	4	5	6	7	9
SPC[c]	3.17[h]	5.91	6.83	6.36	6.79	6.39	6.51	6.25	6.18
APT[d]	2.77	5.79	6.83	6.77	6.74	6.33	6.76	6.29	6.28
M5[e]	2.62	5.91	6.85	6.69	6.77	6.44	6.71	6.56	6.23
STAA[f]	ND[i]	5.90	6.58	6.10	6.40	5.81	5.90	5.77	5.69
VRBG[g]	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] commercial bolgona formulated with 1% (w/w) Alta Mate[R] + 1000 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate before cooking.

[b] inoculated by dipping in a mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* at 10^4 cells.cm^{-2} prior to packaging.

[c] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[d] all purpose tween agar, spiral plated, anaerobic, 25°C for 2 days.

[e] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[f] streptomycin thallous acetate actidione agar, spiral plated, aerobic, 25°C for 2 days.

[g] violet bile red glucose agar, spiral plated, aerobic, 35°C for 1 day.

[h] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[i] none demonstrated.

Table 54. Growth of bacteria in ham formulated with 1% (w/w) Alta Mate^{R} plus 1000 ppm (w/w) eugenol followed by cooking, and stored under vacuum at 8°C (untreated control, uninoculated).

	Untreated (control) - uninoculated										
Media	weeks of storage										
	0	1	2	3	4	5	6	7	9	11	13
SPC[a]	ND[f,g]	ND	ND	ND	ND	ND	ND	ND	ND	>9.00[h]	7.64
APT[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND	>9.00[h]	7.81
M5[c]	ND	ND	ND	ND	ND	ND	ND	ND	ND	>9.00[h]	7.87
STAA[d]	ND	ND	ND	ND	ND	ND	ND	ND	ND	>9.00[h]	ND
VRBG[e]	ND	ND	ND	ND	ND	ND	ND	ND	ND	>9.00[h]	ND

[a] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[b] all purpose tween agar, spiral plated, anaerobic, 25°c for 2 days.

[c] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[d] streptomycin thallous acetate actidione agar, spiral plated, aerobic 25°C for 2 days.

[e] violet bile red glucose agar, spiral plated, aerobic, 35°C for 1 day.

[f] none demonstrated.

[g] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[h] n=2, plated in triplicate.

Table 55. Growth of bacteria in ham formulated with 1% (w/w) Alta Mate^{R} plus 1000 ppm (w/w) eugenol followed by cooking, inoculation with *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* and stored under vacuum at 8°C (untreated, inoculated).

	Untreated - inoculated[a]								
Media	weeks of storage								
	0	1	2	3	4	5	6	7	9
SPC[b]	3.33[g]	6.57	7.61	6.93	7.62	7.52	6.95	6.92	6.82
APT[c]	2.78	7.28	7.33	7.34	7.26	7.31	6.94	6.95	6.93
M5[d]	2.77	7.11	7.52	7.58	7.50	7.47	7.13	7.15	6.96
STAA[e]	ND[h]	7.18	7.25	7.25	7.10	7.07	6.72	6.77	6.62
VRBG[f]	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] inoculated by dipping in a mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* at 10^4 cells.cm^{-2} prior to packaging.

[b] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[c] all purpose tween agar, spiral plated, anaerobic, 25°C for 2 days.

[d] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[e] streptomycin thallous acetate actidione agar, spiral plated, aerobic, 25°C for 2 days.

[f] violet bile red glucose agar, spiral plated, aerobic, 35°C for 1 day.

[g] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[h] none demonstrated.

Table 56. Growth of bacteria in ham formulated with 1% (w/w) Alta Mate[R] plus 1000 ppm (w/w) eugenol followed by cooking, and stored under vacuum at 8°C (treated control, uninoculated).

	Treated (control)[a] - uninoculated										
Media	weeks of storage										
	0	1	2	3	4	5	6	7	9	11	13
SPC[b]	ND[g,h]	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND
APT[c]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND
M5[d]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND
STAA[e]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND
VRBG[f]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND

[a] commercial ham formulated with 1% (w/w) Alta Mate[R] plus 1000 ppm (w/w) eugenol before cooking.

[b] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[c] all purpose tween agar, spiral plated, anaerobic, 25°c for 2 days.

[d] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[e] streptomycin thallous acetate actidione agar, spiral plated, aerobic 25°C for 2 days.

[f] violet bile red glucose agar, spiral plated, aerobic, 35°C for 1 day.

[g] none demonstrated.

[h] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[i] n=2, plated in triplicate.

Table 57. Growth of bacteria in ham formulated with 1% (w/w) Alta Mate[R] plus 1000 ppm (w/w) eugenol followed by cooking, inoculation with *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* and stored under vacuum at 8°C (treated, inoculated).

	Treated[a] - inoculated[b]								
Media	weeks of storage								
	0	1	2	3	4	5	6	7	9
SPC[c]	3.12[h]	6.95	7.29	7.95	7.50	7.14	7.07	5.84	5.58
APT[d]	2.70	7.68	7.33	7.41	7.29	7.05	7.14	5.78	5.66
M5[e]	2.62	7.49	7.49	7.61	7.43	7.09	6.37	5.80	5.52
STAA[f]	ND[i]	7.31	7.07	7.18	6.83	6.61	5.40	4.95	4.28
VRBG[g]	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] commercial ham formulated with 1% (w/w) Alta Mate[R] plus 1000 ppm (w/w) eugenol before cooking.

[b] inoculated by dipping in a mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* at 10^4 cells.cm^{-2} prior to packaging.

[c] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[d] all purpose tween agar, spiral plated, anaerobic, 25°C for 2 days.

[e] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[f] streptomycin thallous acetate actidione agar, spiral plated, aerobic, 25°C for 2 days.

[g] violet bile red glucose agar, spiral plated, aerobic, 35°C for 1 day.

[h] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[i] *none demonstrated.*

Table 58. Growth of bacteria in bologna formulated with 1% (w/w) Alta Mate^{R} + 1000 ppm (w/w) eugenol + 3% (w/w) potassium lactate + 0.4% disodium pyrophosphate followed by cooking, and stored under vacuum at 8°C (untreated control, uninoculated).

	Untreated (control) - uninoculated										
Media	weeks of storage										
	0	1	2	3	4	5	6	7	9	11	13
SPC[a]	ND[f,g]	ND	ND	ND	ND	ND	ND	ND	ND	2.11[h]	5.95
APT[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND	4.02[h]	6.11
M5[c]	ND	ND	ND	ND	ND	ND	ND	ND	ND	3.82[h]	6.47
STAA[d]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[h]	ND
VRBG[e]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[h]	ND

[a] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[b] all purpose tween agar, spiral plated, anaerobic, 25°c for 2 days.

[c] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[d] streptomycin thallous acetate actidione agar, spiral plated, aerobic 25°C for 2 days.

[e] violet bile red glucose agar, spiral plated, aerobic, 35°C for 1 day.

[f] none demonstrated.

[g] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[h] $\log_{10}$ CFU.cm^{-2}, duplicate samples in triplicate.

Table 59. Growth of bacteria in bologna formulated with 1% (w/w) Alta Mate^{R} + 1000 ppm (w/w) eugenol + 3% (w/w) potassium lactate + 0.4% (w/w) disodium pyrophosphate followed by cooking, inoculation with *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* and stored under vacuum at 8°C (untreated, inoculated).

	Untreated - Inoculated[a]								
Media	weeks of storage								
	0	1	2	3	4	5	6	7	9
SPC[b]	2.41[g]	6.98	7.09	7.03	6.96	6.34	6.71	6.86	6.39
APT[c]	3.83	7.13	7.10	7.02	7.09	6.85	6.96	7.04	6.89
M5[d]	2.73	7.22	7.15	7.00	7.05	6.86	6.90	6.91	6.70
STAA[e]	2.41	7.10	6.87	6.88	6.71	P[i]	6.53	6.35	6.32
VRBG[f]	ND[h]	ND	ND	ND	ND	ND	ND	ND	ND

[a] inoculated by dipping in a mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* at 10^4 cells.cm^{-2} prior to packaging.

[b] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[c] all purpose tween agar, spiral plated, anaerobic, 25°C for 2 days.

[d] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[e] streptomycin thallous acetate actidione agar, spiral plated, aerobic, 25°C for 2 days.

[f] violet bile red glucose agar, spiral plated, aerobic, 35°C for 1 day.

[g] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[h] none demonstrated.

[i] STAA plates were improperly prepared and were no good after incubation.

Table 60. Growth of bacteria in bologna formulated with 1% (w/w) Alta Mate[R] plus 1000 ppm (w/w) eugenol + 3% (w/w) potassium lactate + 0.4% (w/w) disodium pyrophosphate followed by cooking, and stored under vacuum at 8°C (treated control, uninoculated).

	Treated (control)[a] - uninoculated										
Media	weeks of storage										
	0	1	2	3	4	5	6	7	9	11	13
SPC[b]	ND[g,h]	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND
APT[c]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND
M5[d]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND
STAA[e]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND
VRBG[f]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND

[a] commercial bolgona formulated with 1% (w/w) Alta Mate[R] plus 1000 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate before cooking.

[b] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[c] all purpose tween agar, spiral plated, anaerobic, 25°c for 2 days.

[d] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[e] streptomycin thallous acetate actidione agar, spiral plated, aerobic 25°C for 2 days.

[f] violet bile red glucose agar, spiral plated, aerobic, 35°C for 1 day.

[g] none demonstrated.

[h] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[i] $\log_{10}$ CFU.cm^{-2}, duplicate samples in triplicate.

Table 61. Growth of bacteria in bologna formulated with 1% (w/w) Alta Mate[R] plus 1000 ppm (w/w) eugenol + 3% (w/w) potassium lactate + 0.4% (w/w) disodium pyrophosphate followed by cooking, inoculation with *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* and stored under vacuum at 8°C (treated, inoculated).

	Treated[a] - inoculated[b]								
Media	weeks of storage								
	0	1	2	3	4	5	6	7	9
SPC[c]	2.52[h]	5.95	6.27	6.54	6.54	6.00	6.25	6.31	6.41
APT[d]	3.81	5.93	6.44	6.50	6.82	6.46	6.48	6.19	6.43
M5[e]	2.41	5.98	6.55	6.64	6.47	6.44	6.47	6.28	6.41
STAA[f]	2.39	5.72	7.26	6.13	5.99	P[j]	5.71	5.34	5.59
VRBG[g]	ND[i]	ND	ND	ND	ND	ND	ND	ND	ND

[a] commercial bolgona formulated with 1% (w/w) Alta Mate[R] + 1000 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate + 3% (w/w) potassium lactate before cooking.

[b] inoculated by dipping in a mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* at 10^4 cells.cm^{-2} prior to packaging.

[c] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[d] all purpose tween agar, spiral plated, anaerobic, 25°C for 2 days.

[e] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[f] streptomycin thallous acetate actidione agar, spiral plated, aerobic, 25°C for 2 days.

[g] violet bile red glucose agar, spiral plated, aerobic, 35°C for 1 day.

[h] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[i] none demonstrated.

[j] STAA plates were improperly prepared and were no good after incubation.

Table 62. Growth of bacteria in ham formulated with 1% (w/w) Alta Mate[R] plus 1000 ppm (w/w) eugenol followed by cooking, and stored under vacuum at 8°C (untreated control, uninoculated).

	Untreated (control) - uninoculated										
Media	weeks of storage										
	0	1	2	3	4	5	6	7	9	11	13
SPC[a]	ND[f,g]	ND	ND	ND	ND	ND	ND	ND	ND	2.41[h]	6.99
APT[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND	3.51[h]	6.98
M5[c]	ND	ND	ND	ND	ND	ND	ND	ND	ND	3.47[h]	7.22
STAA[d]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[h]	ND
VRBG[e]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[h]	ND

[a] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[b] all purpose tween agar, spiral plated, anaerobic, 25°c for 2 days.

[c] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[d] streptomycin thallous acetate actidione agar, spiral plated, aerobic 25°C for 2 days.

[e] violet bile red glucose agar, spiral plated, aerobic, 35°C for 1 day.

[f] none demonstrated.

[g] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[h] $\log_{10}$ CFU.cm^{-2}, duplicate samples in triplicate.

Table 63. Growth of bacteria in ham formulated with 1% Alta Mate[R] plus 1000 ppm (w/w) eugenol followed by cooking, inoculation with *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* and stored under vacuum at 8°C (untreated, inoculated).

	Untreated - Inoculated[a]								
Media	weeks of storage								
	0	1	2	3	4	5	6	7	9
SPC[b]	2.86[g]	7.10	7.23	7.77	7.43	6.68	7.12	7.07	6.96
APT[c]	3.56	7.20	7.37	7.41	7.39	7.17	7.17	7.04	7.02
M5[d]	2.91	7.30	7.61	7.62	7.61	7.47	7.35	7.24	7.23
STAA[e]	2.59	7.13	7.32	7.30	7.22	7.01	7.01	6.86	6.83
VRBG[f]	ND[h]	ND	ND	ND	ND	ND	ND	ND	ND

[a] inoculated by dipping in a mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* at 10^4 cells.cm^{-2} prior to packaging.

[b] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[c] all purpose tween agar, spiral plated, anaerobic, 25°C for 2 days.

[d] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[e] streptomycin thallous acetate actidione agar, spiral plated, aerobic, 25°C for 2 days.

[f] violet bile red glucose agar, spiral plated, aerobic, 35°C for 1 day.

[g] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[h] none demonstrated.

Table 64. Growth of bacteria in ham formulated with 1% (w/w) Alta Mate[R] plus 1000 ppm (w/w) eugenol + 3% (w/w) potassium lactate followed by cooking and stored under vacuum at 8°C (treated control, uninoculated).

	Treated (control)[a] - uninoculated										
Media	weeks of storage										
	0	1	2	3	4	5	6	7	9	11	13
SPC[b]	ND[g,h]	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND
APT[c]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND
M5[d]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND
STAA[e]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND
VRBG[f]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND[i]	ND

[a] commercial ham formulated with 1% (w/w) Alta Mate[R] plus 1000 ppm (w/w) eugenol before cooking.

[b] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[c] all purpose tween agar, spiral plated, anaerobic, 25°c for 2 days.

[d] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[e] streptomycin thallous acetate actidione agar, spiral plated, aerobic 25°C for 2 days.

[f] violet bile red glucose agar, spiral plated, aerobic, 35°C for 1 day.

[g] none demonstrated.

[h] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[i] $\log_{10}$ CFU.cm^{-2}, duplicate samples in triplicate.

Table 65. Growth of bacteria in ham formulated with 1% (w/w) Alta Mate[R] plus 1000 ppm (w/w) eugenol + 3% (w/w) potassium lactate followed by cooking, inoculation with *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* and stored under vacuum at 8°C (treated, inoculated).

	Treated[a] - inoculated[b]								
Media	weeks of storage								
	0	1	2	3	4	5	6	7	9
SPC[c]	2.66[h]	7.01	6.98	7.47	6.72	6.01	6.38	6.30	6.15
APT[d]	3.65	7.09	7.48	7.21	6.69	6.86	6.53	6.26	6.15
M5[e]	2.67	7.14	7.70	7.30	6.62	7.00	6.70	6.14	6.03
STAA[f]	2.77	7.05	7.23	6.76	5.79	P[j]	5.88	5.43	4.92
VRBG[g]	ND[i]	ND	ND	ND	ND	ND	ND	ND	ND

[a] commercial ham formulated with 1% (w/w) Alta Mate[R] plus 1000 ppm (w/w) eugenol before cooking.

[b] inoculated by dipping in a mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* at 10^4 cells.cm^{-2} prior to packaging.

[c] standard plate count agar, spiral plated, anaerobic, 25°C for 2 days.

[d] all purpose tween agar, spiral plated, anaerobic, 25°C for 2 days.

[e] M5 agar, spiral plated, anaerobic, 25°C for 2 days.

[f] streptomycin thallous acetate actidione agar, spiral plated, aerobic, 25°C for 2 days.

[g] violet bile red glucose agar, spiral plated, aerobic, 35°C for 1 day.

[h] $\log_{10}$ CFU.cm^{-2}, triplicate samples in duplicate.

[i] none demonstrated.

[j] STAA plates were improperly prepared and were no good after incubation.

analysed for microbial growth up to week 13, compared to the inoculated untreated and treated meats where sampling was halted after week 9. These results were similar to the previous combinations where Alta Mate® and eugenol were used at lower concentrations.

It is disappointing that the antimicrobial agents chosen from preliminary tests were unsuccessful in the present meat model systems. To date there are no scientific publications on the inhibitory effects of Alta Mate®, and therefore comparisons with other research data are not possible. However, the manufacturer insists that Alta Mate® is a good antimicrobial agent and that it has worked successfully in their laboratories.

Eugenol, alone and in combination with other antimicrobial agents, in test broth has shown some promise in its ability to delay the growth of spoilage microorganisms from the work of other researchers (Blaszyk and Holley, 1998). As well, published reports have shown that eugenol is effective in inhibiting a broad range of microorganisms, both Gram-positive and Gram-negative bacteria in test agar and broths (Suresh et al., 1992; Jay and Rivers, 1984; Moleyar and Narasimham, 1992; Shelef, 1983; Blaszyk and Holley, 1998). Therefore, it was discouraging to find that eugenol was unable to inhibit *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* in bologna and ham sausages.

Disodium pyrophosphate (0.4%) was unable to help inhibit both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* in bologna and ham sausage samples. Phosphates in general have been frequently used in the food industry as processing aids or additives, and have recently been evaluated for their antimicrobial properties due to their chelating properties. Marcy et al. (1988) found that 0.4% disodium pyrophosphate

decreased the viable number of several bacteria compared to the control in cooked vacuum-packaged pork sausages held at refrigerated temperatures for 21 d. Differences of two log cycles for both the mesophilic and the facultative anaerobic bacteria were reported.

Results of this study indicate that there was no increase in inhibition of *Lc. mesenteroides* ssp. *mesenteroides* or *Lb. sakei* with the addition of 3% potassium lactate. Therefore, the potassium lactate did not sufficiently delay the growth of either test organism. This was unfortunate since Bradford et al. (1993a and 1993b) found that the addition of 2% and 3% potassium lactate to typical fresh pork sausage stored at 5-7°C resulted in reduced populations of psychrotrophs and coliforms. They concluded that the reduction in microbial numbers was probably due to one or both of two factors; 1) the ability of undissociated acids such as potassium lactate to cross cell membranes, dissociate and acidify the cell interior, and 2) lactates may reduce water activity which would result in decreased microbial numbers. Weaver and Shelef (1993) also found that numbers of *L. monocytogenes* cells were suppressed in pork liver samples containing 2% and 3% potassium lactate that were held at 5°C for 50 d.

It should be noted that results from bacterial analyses of meat samples were not analysed statistically because the only differences between treatments were clear. These were with uninoculated treated and untreated meats near the end of storage periods where adventitious bacteria grew in untreated products.

It was interesting to monitor both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* throughout the meat trials on M5 media. It was found that both target organisms

were able to survive throughout the testing period with *Lb. sakei* generally dominating throughout the meat trials (Tables 66-69).

Table 66. Number[a] of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* grown on M5 plates[bc] from meat formulated with 0.5% (w/w) Alta Mate[R] plus 400 ppm (w/w) eugenol plus 0.4% (w/w) disodium pyrophosphate and stored at 8°C for up to 5 weeks.

Organism	Bologna - untreated weeks of storage				Bologna - treated weeks of storage			
	0	1	3	5	0	1	3	5
Lb. sakei	3.15	ND[d]	6.28	6.95	2.92	ND	6.65	6.85
Lc. mesenteroides	2.85	ND	6.28	6.85	2.65	ND	6.76	6.58
	Ham - untreated weeks of storage				Ham - treated weeks of storage			
	0	1	3	5	0	1	3	5
Lb. sakei	3.30	ND	ND	5.94	3.08	ND	6.95	6.81
Lc. mesenteroides	2.88	ND	ND	6.05	2.88	ND	7.06	6.88

[a] $\log_{10}$ CFU.cm^{-2}.

[b] plates incubated at 25°C for 2 days.

[c] n=6.

[d] the exact proportion of each organism not determined, however, *Lb. sakei* dominated.

Table 67. Number[a] of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* grown on M5 plates[bc] from meat formulated with 0.5% (w/w) Alta Mate[R] plus 350 ppm (w/w) eugenol plus 0.4% (w/w) disodium pyrophosphate plus 3% (w/w) potassium lactate and stored at 8°C for up to 5 weeks.

Organism	Bologna - untreated weeks of storage				Bologna - treated weeks of storage			
	0	1	3	5	0	1	3	5
Lb. sakei	2.50	ND[d]	7.01	6.92	2.71	ND	6.41	6.11
Lc. mesenteroides	2.50	ND	6.95	6.81	2.65	ND	6.28	6.11
	Ham - untreated weeks of storage				Ham - treated weeks of storage			
	0	1	3	5	0	1	3	5
Lb. sakei	2.92	ND	6.41	6.28	2.50	ND	7.17	6.98
Lc. mesenteroides	2.81	ND	6.41	6.58	2.58	ND	7.35	7.19

[a] $\log_{10}$ CFU.cm^{-2}.

[b] plates incubated at 25°C for 2 days.

[c] n=6.

[d] the exact proportion of each organism not determined, however, *Lb. sakei* dominated.

Table 68. Number[a] of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* grown on M5 plates[bc] from meat formulated with 1% (w/w) Alta Mate[R] plus 1000 ppm (w/w) eugenol and stored at 8°C for up to 9 weeks.

Organism	Bologna - untreated weeks of storage								
	0	1	2	3	4	5	6	7	9
Lb. sakei	2.76	ND[e]	7.24	7.06	7.01	7.17	6.85	6.81	6.65
Lc. mesenteroides	1.81	ND	6.41	6.41	6.41	6.28	6.50	6.28	6.11
	Bologna[d] - treated weeks of storage								
	0	1	2	3	4	5	6	7	9
Lb. sakei	2.58	ND	6.81	6.58	6.65	6.28	6.65	6.50	6.11
Lc. mesenteroides	1.11	ND	6.11	6.11	6.11	5.81	5.81	5.81	5.81
	Ham - untreated weeks of storage								
	0	1	2	3	4	5	6	7	9
Lb. sakei	2.71	ND	7.42	7.47	7.37	7.32	6.85	7.06	6.76
Lc. mesenteroides	1.81	ND	6.85	6.92	6.88	6.92	6.65	6.50	6.50
	Ham - treated weeks of storage								
	0	1	2	3	4	5	6	7	9
Lb. sakei	2.58	ND	7.37	7.52	7.32	6.98	6.28	5.65	5.41
Lc. mesenteroides	1.81	ND	6.85	6.88	6.81	6.41	5.81	5.25	4.88

[a] $\log_{10}$ CFU.cm^{-2}.

[b] plates incubated at 25°C for 2 days.

[c] n=6.

[d] formulated with the addition of 0.4% (w/w) disodium pyrophosphate.

[e] the exact proportion of each organism not determined, however, *Lb. sakei* dominated.

Table 69. Number[a] of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* grown on M5 plates[b,c] from meat formulated with 1% (w/w) Alta Mate[R] plus 1000 ppm (w/w) eugenol plus 3% (w/w) potassium lactate and stored at 8°C for up to 9 weeks.

Organism	Bologna - untreated weeks of storage								
	0	1	2	3	4	5	6	7	9
Lb. sakei	2.41	7.11	7.04	6.85	6.85	6.65	6.76	6.71	6.58
Lc. mesenteroides	1.81	6.58	6.58	6.50	6.58	6.50	6.41	6.50	6.11
	Bologna[d] - treated weeks of storage								
	0	1	2	3	4	5	6	7	9
Lb. sakei	2.28	ND[e]	6.41	6.50	6.28	6.28	6.50	6.28	6.28
Lc. mesenteroides	1.81	ND	6.11	6.11	6.11	6.11	5.81	5.81	5.81
	Ham - untreated weeks of storage								
	0	1	2	3	4	5	6	7	9
Lb. sakei	2.50	7.19	7.47	7.49	7.46	7.22	7.19	7.08	7.06
Lc. mesenteroides	2.28	6.65	7.08	7.06	7.08	7.11	7.01	6.76	6.71
	Ham - treated weeks of storage								
	0	1	2	3	4	5	6	7	9
Lb. sakei	3.5	7.04	7.58	7.13	6.41	6.85	6.50	6.11	7.17
Lc. mesenteroides	2.28	6.41	7.08	6.76	6.11	6.50	6.28	5.81	6.92

[a] $\log_{10}$ CFU.cm^{-2}.

[b] plates incubated at 25°C for 2 days.

[c] n=6.

[d] formulated with the addition of 0.4% (w/w) disodium pyrophosphate.

[e] the exact proportion of each organism not determined, however, *Lb. sakei* dominated.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

Initial trials with liquid smoke, horseradish oil, eugenol, potassium lactate, Alta® 2341, polyphosphoric acid and Lauricidin® conducted in test tubes containing MRS broth resulted in inhibition of both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei*. It was concluded that the polyphosphoric acid with a pH ~4.80 was responsible for the inhibition and that the other antimicrobials added had little or no effect on inhibition.

Growth inhibition tests revealed that Lauricidin® and Alta Mate® at 25°C were not a compatible combination since white pellets (or viscous liquid) were produced at the bottom of the microtitre plate wells which probably affected antimicrobial activity. It was concluded that Alta Mate® and Lauricidin® interacted to produce the insoluble material, but this has not been further studied.

Agar spot, well diffusion, and disc assay tests failed to provide significant information on the antimicrobials tested. Problems ranged from the volatility of some antimicrobial agents used to the inability of the antimicrobials to diffuse through the agar. These tests also failed to validate results obtained from other such tests like spiral gradient endpoint (SGE) antimicrobial susceptibility and the broth tube tests.

Antimicrobial agents such as Lauricidin®, disodium pyrophosphate, Alta Mate®, Alta® 2341, eugenol, EDTA, phosphate glass, and potassium lactate were tested alone and in combination against *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* using the SGE antimicrobial susceptibility test at 8°C and 25°C. The tests were conducted on MRS agar supplemented with 2.5% NaCl and 100 ppm $NaNO_2$ to mimic, as close as possible,

conditions in cured meats. The dominant antimicrobial agent that seemed to be most effective against the microorganisms was probably Alta Mate®. However, preliminary tests were not done using Alta Mate® alone (in broth trials, other inhibitors used increased the effectiveness of Alta Mate®). Problems arose with the use of Lauricidin® during testing because it became insoluble (formed a thick white viscous liquid) either within the stylus of the spiral plater or on the MRS agar at 8°C. Therefore, further tests were halted due to inaccurate test results. It was concluded that Alta Mate®, eugenol, disodium pyrophosphate and potassium lactate were most effective against both organisms and were chosen for use in bologna and ham samples.

The results in the meat trials revealed that combinations of 0.5-1% (w/w) Alta Mate®, 350, 400 and 1000 (w/w) ppm eugenol, 0.4% (w/w) disodium pyrophosphate and 3% (w/w) potassium lactate did not inhibit the growth of inoculated *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei*. However, these combinations did inhibit the natural microflora present. It was difficult to determine whether eugenol was able to inhibit either *Lc. mesenteroides* ssp. *mesenteroides* or *Lb. sakei* in meat trials because eugenol is somewhat volatile and could have volatilised during cooking. This was unlikely though, because meats were vacuum-packaged before cooking and there was no indication by smell that this happened. However, preliminary tests showed that eugenol was an effective antimicrobial agent and appeared to interact positively with Alta Mate® against both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei*.

When ham sausages were prepared, it was found, visually, that the colour of the emulsions was slightly bleached when the antimicrobials were added and mixed thoroughly with batter, cooked and stored at 8°C. This may not be appealing to

consumers since the treated ham was visually more homogenous in appearance, much like light coloured bologna. The extra manipulation during treatment addition tended to reduce the size and obscure the chunks of ham and veins of fat that are usually noticeable in chopped ham slices used for sandwich meat. It is likely that consumers would consider the appearance of treated ham less desirable. However, the addition of antimicrobials to bologna did not alter the colour of the emulsion visually. When the Miniscan spectrocolourimeter was used to determine colour accurately, it was found that there were significant differences in colour when both bologna and ham samples were treated with the antimicrobials. As well, the addition of 3% (w/w) potassium lactate to bologna and ham samples treated with 1% (w/w) Alta Mate® plus 1000 ppm (w/w) eugenol caused the bologna samples to become slightly darker and ham samples to initially become lighter in colour compared to samples without any treatment. Upon 8°C storage, a bleaching effect occurred in ham treatments that may have been the result of the extra chopping step used in preparation of the treated ham samples, or it may have been due to addition of Alta Mate® and/or eugenol.

Both pH and a_w were studied during the course of this work. As expected the pH of inoculated untreated and treated bologna and ham sausage samples decreased due to the presence of both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei*. There was a very slow decline in pH of uninoculated untreated and treated sausage samples which was also expected since only the natural microflora was present. The a_w of ham was significantly lower when 3% (w/w) potassium lactate was added to samples with combinations involving Alta Mate®, eugenol, and disodium pyrophosphate. Bologna a_w

was slightly lower with the addition of 3% (w/w) potassium lactate but the difference was not significant.

Visual and odour analyses were also carried out at each week during the shelf-life period. It was concluded the samples did not differ from week to week visually, however, the odour increased as the storage interval increased and was probably related to the increased levels of *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* (or spoilage organisms).

Throughout the meat trials, both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* found in the bologna and ham samples were plated on M5 medium. Both *Lc. mesenteroides* ssp. *mesenteroides* and *Lb. sakei* contributed to spoilage, with *Lb. sakei* being the dominant microorganism in combinations consisting of 1% (w/w) Alta Mate®, 1000 ppm (w/w) eugenol, 0.4% (w/w) disodium pyrophosphate and 3% (w/w) potassium lactate. In samples treated with 0.5% (w/w) Alta Mate®, 350 or 400 ppm (w/w) eugenol, 0.4% (w/w) disodium pyrophosphate and 3% (w/w) potassium lactate *Lb. sakei* was not always the dominant microorganism.

Recommendations for further research based on results from this study include:

- Apply antimicrobial agent(s) to an edible gel or coatings, or embed into packaging film which may provide barrier/hurdles to reduce the incidence of pathogenic or spoilage bacteria on surfaces of both raw and cooked meat products.
- Explore other antimicrobial agents such as chitin, chitosan and their derivatives in: preliminary tests; edible films and coatings; meat model system(s).

- Apply volatile antimicrobial(s) to a "soak" pad that is located between the oxygen barrier film and the raw meat product to limit the surface bacteria and to prolong the shelf-life of these products.
- Explore other possibilities in testing Lauricidin® plus other lipophilic natural antimicrobial plant extracts and spice oils in preliminary tests that would be applicable in meat model systems.
- Explore the possibility of treating meat products with bacteriocins that have an impact on spoilage microorganisms, especially lactobacilli.
- Explore the idea of combining natural antimicrobials and thermal treatments (performed after packaging) that would be applicable in meat model systems.

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Appendices

Appendix 1. Chemicals

Chemicals	Manufacturers
Trypticase peptone	BBL - Becton Dickinson and Co., Cockeysville, MD
Yeast extract	Sigma-Aldrich Canada, Oakville, ON
Agar granulated	BBL - Becton Dickinson and Co., Cockeysville, MD
Tween 80 (polysorbate 80 USP)	Fisher Scientific Canada Inc., Nepean, ON
Fructose ($C_6H_{12}O_6$)	Mallinckrodt Specialty Chemicals Co., Paris, KT
D-(+)-glucose ($C_6H_{12}O_6$)	Sigma-Aldrich Canada, Oakville, ON
Potassium dihydrogen phosphate (KH_2PO_4)	Mallinckrodt Specialty Chemicals Co., Paris, KT
Dipotassium hydrogen phosphate (K_2HPO_4) (dibasic)	Mallinckrodt Specialty Chemicals Co., Paris, KT
Magnesium sulphate ($MgSO_4.7H_2O$)	Mallinckrodt Specialty Chemicals, Co., Paris, KT
Manganese sulfate ($MnSO_4.H_2O$)	Mallinckrodt Specialty Chemicals, Co., Paris, KT
Sodium hydroxide (NaOH)	Mallinckrodt Specialty Chemicals, Co., Paris, KT
Hydrogen peroxide (3% U.S.P.) (H_2O_2)	Life Brand - Extra Foods Grocery, Winnipeg, MB
Sodium chloride (NaCl)	Sigma-Aldrich Canada, Oakville, ON
Sodium nitrite ($NaNO_2$)	J. T. Baker Chemical Co., Phillipsburg, NJ
Phytic acid	Sigma-Aldrich Canada, Oakville, ON

Appendix 1. Chemicals (*continued*)

Chemicals	Manufacturers
L-cysteine hydrochloide monohydrate ($C_3H_7NO_2S.HCl.H_2O$)	Sigma-Aldrich Canada, Oakville, ON
Whey protein (Lot # R0031)	Inovatech, Winnipeg, MB
Calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)	Sigma-Aldrich Canada, Oakville, ON
Bromcresol green sodium ACS reagent ($C_{21}H_{13}Br_4O_5SNa$)	Sigma-Aldrich Canada, Oakville, ON
Light mineral oil (Paraffin oil, light)	Mallinckrodt Specialty Chemicals, Co., Paris, KT
Disodium ethylenediamintetracetate (EDTA) ($Na_2C_{10}H_{14}O_8N_2.2H_2O$)	Fisher Scientific Canada Inc., Nepeean, ON
Morpholinoethane sulphonic acid (MES enzyme grade)	Fisher Scientific Canada Inc., Nepean, ON
Disodium pyrophosphate, practical grade ($Na_2H_2P_2O_7$)	Sigma-Aldrich Canada, Oakville, ON
Sodium tripolyphosphate ($Na_5P_3O_{10}$)	Fisher Scientific Canada Inc., Nepean, ON
Eugenol ($C_{10}H_{12}O_2$) (2-methoxy-4-[2-propenyl]phenol)	Sigma-Aldrich Canada, Oakville, ON
Allyl isothiocyanate (95%) ($H_2C=CHCH_2NCS$)	Sigma-Aldrich Canada, Oakville, ON
Propylene glycol, USP/FCC	Fisher Scientific Canada Inc., Nepean, ON
Phosphate glass, practical grade ($Na_{15}P_{13}O_{40}$-$Na_{20}P_{18}O_{55}$)	Sigma-Aldrich Canada, Oakville, ON

Appendix 1. Chemicals (*continued*)

Chemicals	Manufacturers
Cycloheximide from *Streptomyces griseus* ($C_{15}H_{23}NO_4$)	Sigma-Aldrich Canada, Oakville, ON
Potassium phosphate (KH_2PO_4) (monobasic-anhydrous)	Sigma-Aldrich Canada, Oakville, ON
Thallium (I) acetate ($TIC_2H_3O_2$)	Sigma-Aldrich Canada, Oakville, ON
Streptomycin sulfate	Sigma-Aldrich Canada, Oakville, ON
D-pantothenic acid hemicalcium ($C_9H_{16}NO_5.1/2Ca$)	Sigma-Aldrich Canada, Oakville, ON
Glycerol ACS reagent	Sigma-Aldrich Canada, Oakville, ON
Tryptone peptone	Difco Laboratories, Detroit, MI
Lauricidin^R (Lot no. 71112)	Technology Exchange, Inc., Galena, IL
Alta^R 2341 (code 45-046)	Rector Foods Ltd., Mississauga, ON
Alta Mate^R (manuf. Date 10 Jun 99)	Rector Foods Ltd., Mississauga, ON
Potassium lactate (85% v/v)	Canada Compound Western Ltd., Winnipeg, MB
Polyphosphoric acid (H_3PO_4)	Sigma-Aldrich Canada, Oakville, ON
Aro-smoke P-50 liquid smoke	Red Arrow Products Company, Inc., Manitowoc, WI
Potassium hydroxide (KOH)	Mallinckrodt Specialty Chemicals Co., Paris, KT
Immersion oil	Fisher Scienftific Canada Inc., Nepean, ON
Ethanol (95%)	Commercial Alcohols Inc., Toronto, ON

Appendix 2. Media and reagents

Media/reagents	Manufacturers/References
Lactobacilli MRS broth (dehydrated)	Difco Laboratories, Detroit, MI
All purpose tween broth (APT)	BBL - Becton Dickinson and Co., Cockeysville, MD
All purpose tween agar (APT)	BBL - Becton Dickinson and Co., Cockeysville, MD
M5 agar	Zuniga et al., 1993
Standard methods agar (SPC)	BBL - Becton Dickinson and Co., Cockeysville, MD
Violet red bile agar (VRB)	BBL - Becton Dickinson and Co., Cockeysville, MD
Streptomycin thallous acetate actidione agar (STAA)	Gill and Greer, 1993
Agar granulated	BBL - Becton Dickinson and Co., Cockeysville, MD

Appendix 3. Supplies and instruments

Supplies/instruments	Manufacturers
Seward medical stomacher bags (177x304 mm) and Stomacher lab-blender (model 400)	Seward laboratory, London, UK
pH Accumet Basic Microprocessor pH/mV*i* C meter	Fisher Scientific Canada Inc., Nepean, ON
0.05~10, 0.1~10, 1~100, and 100~1000 ul Eppendorf reference pipetters and pipette tips	Fisher Scientific Canada Inc., Nepean, ON
Vortex Genie-2 mixer	Fisher Scientific Canada Inc., Nepean, ON
BBL GasPak CO_2 atmosphere anaerobic system (including jars, disposable GasPak Plus envelops with palladium catalyst and disposable anaerobic indicators)	Becton Dickinson and Co., Cockeysville, MD
Quebec dark-field colony counter	American Optical Scientific Instrument Division, Buffalo, NY
Microscope slides and cover glass	Fisher Scientific Canada Inc., Nepean, ON
API 20 E System kit	bioMerieux Vitek, Inc., Hazelwood, MO
Deli #1 high-O_2 barrier pouches (polyvinylediene chloride (PVDC)) (25 x 35 cm)	Winpak Inc., Winnipeg, MB
Bizerba vacuum packager (model GM 2002)	Bizerba Canada, Inc., Mississauga, ON
Zeiss universal research microscope (model D-7082)	Carl Zeiss Inc., Oberkochen, Germany

Appendix 3. Supplies and instruments *(continued)*

Supplies/instruments	Manufacturers
96-well Falcon 3072 Microtest III tissue culture plates	Becton Dickinson and Co., Lincoin Park, NJ
Test tubes (20x125 mm)	Fisher Scientific Canada Inc., Nepean, ON
Cotton swab applicators	Fisher Scientific Canada Inc., Nepean, ON
Petri Dishes (100x15 mm and 150x15 mm)	Fisher Scientific Canada Inc., Nepean, ON
Autoclave Bags (8x12 cm and 12x24 cm)	Fisher Scientific Canada Inc., Nepean, ON
BBL-Blank sterile discs (6.35 mm in diameter)	Fisher Scientific Canada Inc., Nepean, ON
Small latex NDPR texture gloves	Fisher Scientific Canada Inc., Nepean, ON
VWR polystyrene beaker cups (5 ml)	VWR Scientific of Canada Ltd., London, ON
Sterile indicator tape (3/4x500)	Fisher Scientific Canada Inc., Nepean, ON
Magna nylon membrane filters (25 mm with 0.22 um pore size)	Fisher Scientific Canada Inc., Nepean, ON
Laminar flow hood (model #V6MW97T)	Canadian Cabinets Company, Ltd., Nepean, ON
10cc. Syringe	Becton Dickinson and Co., Lincoin Park, NJ
Corning hot plate stirrer (model PC-351)	Fisher Scientific Canada Inc., Nepean, ON

Appendix 3. Supplies and instruments *(continued)*

Supplies/instruments	Manufacturers
Anaerobic incubator chamber (model 3640-6) with CO_2	National Applicance Co., Portland, Oregon
gas and mixture of 30:70 CO_2 and N_2 gas	Welders Supplies Ltd., Winnipeg, MB
Rotary shaker (model OS31)	Fermentation Design Inc., Allentown, PA
New Brunswick scientific rotary shaker (model G-33)	New Brunswick Scientific, New Brunswick, N.J.
Hog rings (3/8")	Canada Compound Western Ltd., Winnipeg, MB
Student grade Whatman filter paper (12.5 cm)	Fisher Scientific Canada Inc., Nepean, ON
Miniscan spectrocolourimeter (model MS-4500L) with plexy glass plates	Hunter Associates Lab Inc., Reston, Virginia
Hobart mixer (model A-200)	The Hobart Manufacturing Co., Troy, OH
Groen steam kettles (models D-10 and D-30)	Groen Division Corporation, Elk Grove Villagn, IL
Sausage stuffer (9 litre) (model FD. 29A 38)	F. Dick Gmbh, Germany
Liquid petrolatum spray (U.S.P.)	The Haynes Manufacturing Co., Cleveland, OH
Autoplater^R 4000, computer system with scanner and CASBA 4 software	Spiral Biotech, Inc., Bethesda, MD

Appendix 3. Supplies and instruments *(continued)*

Supplies/instruments	Manufacturers
Microwell titre plate reader (model 450) and software (version 4.2, single wave length at 655 nm)	Bio-Rad Laboratories, Hercules, CA
Food chopper (model 84142)	Food Cutter, Don Mills, ON
Casing clipper	Republic Fastener Products Corp.
Dry-type bacteriological countertop incubator (model 100A)	Blue M Electric Co., Blue Island, IL
CO_2 countertop incubator (model 3029)	Forma Scientific, Marietta, OH
Screw cap tubes	Fisher Scientific Canada, Nepean, ON
Pasteur pipettes	VWR Scientific of Canada Ltd., London, ON
Decagon Aw machine (model CX-1) with plastic sample cups	Decagon Devices Inc., Pullman, WA
Brinkmann multichannel transferpette pipetter (5-200 ul)	Fisher Scientific Canada, Nepean, ON
JMP start statistics software	SAS Institute Inc., Belmont, CA
Chopped ham and bologna meat	Maple Leaf Meats Winnipeg Ltd., Winnipeg, MB
Hog casings (28-30 mm)	Canada Compound Western Ltd., Winnipeg, MB
Kim wipes	Fisher Scientific Canada, Nepean, ON
Twist stopper (model M66-8)	Fisher Scientific Canada, Nepean, ON
Vacu-guard filters	Fisher Scientific Canada, Neapean, ON

Appendix 4. Standard procedures for LAB strain maintenance (Adopted from G. E. Millard, Centre for Food & Animal Research, Agriculture and Agri-Food Canada, Ottawa).

1. Grow culture 24-48 hr. using MRS broth anaerobically at 25°C.

2. Streak for purity. Examine for morphology, Gram reaction (3% KOH), catalase (3% H_2O_2). Re-isolate back to broth for a second period of growth.

3. Centrifuge the second culture.

4. Decant the supernatant and resuspend the pellet in 1.5 ml fresh broth.

5. Add 0.75 ml filter sterilized glycerol (50% v/v) and mix thoroughly.

6. Add 0.3 ml of the final suspension to each of six labelled cryogenic vials.

7. Freeze at -85°C.

8. Check resuscitation of one vial after more than 30 days storage, and if successful store remaining vials in coded containers at -85°C indefinitely.

Appendix 5. Odour development of bologna and ham samples with and without 0.5% (w/w) Alta Mate^{R} + 400 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate during storage at 8°C.

Treatment/Week(s)	0	1	3	5	9
Bologna-untreated-uninoculated					
Bologna-untreated-inoculated					N/A
Bologna-treated-uninoculated					
Bologna-treated-inoculated				X[a]	N/A
Ham-untreated-uninoculated					
Ham-untreated-inoculated					N/A
Ham-treated-uninoculated					
Ham-treated-inoculated				X	N/A

X sour odour present upon opening bag.

[a] vacuum lost within bag prior to opening.

N/A not available; inoculated samples were spoiled at week 5, therefore, the bags were not analysed for odour.

Appendix 6. Odour development of bologna and ham samples with and without 0.5% (w/w) Alta Mate^{R} + 350 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate + 3% (w/w) potassium lactate during storage at 8°C.

Treatment/Week(s)	0	1	3	5	9
Bologna-untreated-uninoculated				[a]	
Bologna-untreated-inoculated				[a]	N/A
Bologna-treated-uninoculated				[a]	
Bologna-treated-inoculated				[a]	N/A
Ham-untreated-uninoculated				[a]	
Ham-untreated-inoculated				[a]	N/A
Ham-treated-uninoculated				[a]	
Ham-treated-inoculated				X[a]	N/A

X sour odour present upon opening bag.

[a] vacuum lost within bag prior to opening.

N/A not available; inoculated samples were spoiled at week 5, therefore, the bags were not analysed for odour.

Appendix 7. Odour development of bologna and ham samples with and without 1% (w/w) Alta Mate^{R} + 1000 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate during storage at 8°C.

Treatment/Week(s)	0	1	2	3	4	5	6	7	9	11	13
Bologna-untreated-uninoculated											
Bologna-untreated-inoculated				[a]	[a]	[a]	X[a]	X[a]	X[a]	N/A	N/A
Bologna-treated-uninoculated											X
Bologna-treated-inoculated				X[a]	X[a]	X[a]	X[a]	X[a]	X[a]	N/A	N/A
Ham-untreated-uninoculated											
Ham-untreated-inoculated			X	X[a]	[a]		[a]	[a]	[a]	N/A	N/A
Ham-treated-uninoculated											X
Ham-treated-inoculated					X	[a]	X[a]	X[a]	X[a]	N/A	N/A

X sour odour present upon opening bag.

[a] vacuum lost within bag prior to opening.

N/A not available; inoculated samples were spoiled at week 9, therefore, the bags were not analysed for odour.

Appendix 8. Odour development of bologna and ham samples with and without 1% (w/w) Alta Mate^{R} + 1000 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate + 3% (w/w) potassium lactate during storage at 8°C.

Treatment/Week(s)	0	1	2	3	4	5	6	7	9	11	13
Bologna-untreated-uninoculated											
Bologna-untreated-inoculated				[a]	X[a]	X	X[a]	X[a]	X[a]	N/A	N/A
Bologna-treated-uninoculated											X
Bologna-treated-inoculated				X	X[a]	X	X[a]	X[a]	X[a]	N/A	N/A
Ham-untreated-uninoculated											
Ham-untreated-inoculated			X[a]	[a]	X[a]	X	[a]	[a]	[a]	N/A	N/A
Ham-treated-uninoculated											
Ham-treated-inoculated				X		X[a]	X[a]	X[a]	X[a]	N/A	N/A

X sour odour present upon opening bag.

[a] vacuum lost within bag prior to opening.

N/A not available; inoculated samples were spoiled at week 9, therefore, the bags were not analysed for odour.

Appendix 9. Changes in bologna pH following formulation with 0.5% (w/w) Alta Mate^{R} + 400 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate during storage at 8°C under vacuum.

	Bologna				
Week	0	1	3	5	9
Treatment					
Control[a] un-inoc.	6.28[b]	6.38	6.53	6.27	5.51
Control inoc.[c]	6.31	5.78	5.43	5.38	
Treated un-inoc.	6.30	6.37	6.52	6.52	6.37
Treated inoc.	6.31	5.86	4.78	4.53	

[a] commercial formulation with no additions.

[b] n=2.

[c] inoculated with an equal mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* cells to yield 3 log CFU.cm^{-2}.

Appendix 10. Changes in ham pH following formulation with 0.5% (w/w) Alta Mate^{R} + 400 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate during storage at 8°C under vacuum.

	Ham				
Week	0	1	3	5	9
Treatment					
Control[a] un-inoc.	6.50[b]	6.53	6.65	6.63	6.46
Control inoc.[c]	6.46	6.23	5.77	5.54	
Treated un-inoc.	6.49	6.49	6.57	6.58	6.44
Treated inoc.	6.44	5.75	4.98	4.98	

[a] commercial formulation with no additions.

[b] n=2.

[c] inoculated with an equal mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* cells to yield 3 log CFU.cm^{-2}.

Appendix 11. Changes in bologna pH following formulation with 0.5% (w/w) Alta Mate^{R} + 350 ppm (w/w) eugenol + 3% (w/w) potassium lactate + 0.4% (w/w) disodium pyrophosphate during storage at 8°C under vacuum.

	Bologna				
Week	0	1	3	5	9
Treatment					
Control[a] un-inoc.	6.32[b]	6.62	6.54	6.43	6.36
Control inoc.[c]	6.36	5.88	5.37	5.11	
Treated un-inoc.	6.11	6.30	6.29	6.26	6.24
Treated inoc.	6.15	6.18	5.13	5.06	

[a] commercial formulation with no additions.

[b] n=2.

[c] inoculated with an equal mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* cells to yield 3 log CFU.cm^{-2}.

Appendix 12. Changes in ham pH following formulation with 0.5% (w/w) Alta Mate^{R} + 350 ppm (w/w) eugenol + 3% (w/w) potassium lactate + 0.4% (w/w) disodium pyrophosphate during storage at 8°C under vacuum.

	Ham				
Week	0	1	3	5	9
Treatment					
Control[a] un-inoc.	6.59[b]	6.75	6.47	6.17	6.11
Control inoc.[c]	6.60	6.44	5.77	5.49	
Treated un-inoc.	6.53	6.67	6.60	6.53	6.44
Treated inoc.	6.51	6.21	5.17	5.05	

[a] commercial formulation with no additions.

[b] n=2.

[c] inoculated with an equal mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* cells to yield 3 log CFU.cm^{-2}.

Appendix 13. Changes in bologna pH following formulation with 1% (w/w) altamate^{R} + 1000 ppm (w/w) eugenol + 0.4% (w/w) disodium pyrophosphate during storage at 8°C under vacuum.

	Bologna										
Week	0	1	2	3	4	5	6	7	9	11	13
Treatment											
Control uninoc.	6.64[a]	6.55	6.51	6.44	6.45	6.40	6.45	6.46	6.47	6.42[c]	6.41
Control inoc.[b]	6.65	6.28	5.26	5.09	4.73	4.44	4.45	4.65	4.46		
Treated uninoc.	6.31	6.14	6.27	6.23	6.24	6.21	6.19	6.24	6.20	6.25[c]	6.26
Treated inoc.	6.32	6.20	5.73	4.82	4.69	4.50	4.34	4.38	4.24		

[a] n=3.

[b] inoculated with an equal mixture of *Lb. sakei* and *Lc. mesenteroides* cells to yield 3 log CFU.cm^{-2}.

[c] n=2.

Appendix 14. Changes in ham pH following formulation with 1% (w/w) altamate^{R} + 1000 ppm (w/w) eugenol during storage at 8°C under vacuum.

	Ham										
Week	0	1	2	3	4	5	6	7	9	11	13
Treatment											
Control[a] uninoc.	6.69[b]	6.60	6.58	6.51	6.52	6.48	6.41	6.47	6.47	6.06[d]	6.06
Control inoc.[c]	6.73	6.17	5.09	5.00	5.17	4.97	4.98	4.97	4.93		
Treated uninoc.	6.72	6.60	6.59	6.52	6.52	6.47	6.44	6.49	6.46	6.52[d]	6.50
Treated inoc.	6.71	6.51	6.21	6.09	6.06	5.43	4.68	4.49	4.30		

[a] commercial formulation with no additions.

[b] n=3.

[c] inoculated with an equal mixture of *Lb. sakei* and *Lc. mesenteroides* cells to yield 3 log CFU.cm^{-2}.

[d] n=2.

Appendix 15. Changes in bologna pH following formulation with 1% (w/w) Alta Mate^{R} + 1000 ppm (w/w) eugenol + 3% (w/w) potassium lactate + 0.4% (w/w) disodium pyrophosphate during storage at 8°C under vacuum.

	Bologna										
Week	0	1	2	3	4	5	6	7	9	11	13
Treatment											
Control uninoc.	6.49[a]	6.47	6.48	6.46	6.39	6.38	6.53	6.47	6.43	6.43[c]	6.42
Control inoc.[b]	6.48	6.15	5.28	4.87	4.95	4.69	4.75	4.88	4.58		
Treated uninoc.	6.22	6.20	6.17	6.19	6.17	6.18	6.21	6.19	6.23	6.24[c]	6.22
Treated inoc.	6.14	6.15	5.97	5.56	5.09	5.09	5.04	4.95	4.97		

[a] n=3.

[b] inoculated with an equal mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* cells to yield 3 log CFU.cm^{-2}.

[c] n=2.

Appendix 16. Changes in ham pH following formulation with 1% (w/w) Alta Mate[R] + 1000 ppm (w/w) eugenol + 3% (w/w) potassium lactate during storage at 8°C under vacuum.

	Ham										
Week	0	1	2	3	4	5	6	7	9	11	13
Treatment											
Control[a] uninoc.	6.54[b]	6.53	6.52	6.50	6.44	6.41	6.40	5.21	6.49	6.50[d]	6.49
Control inoc.[c]	6.54	5.90	5.03	5.04	4.99	4.99	5.00	4.95	5.07		
Treated uninoc.	6.53	6.53	6.52	6.50	6.46	6.46	6.48	6.45	6.5	6.53[d]	6.52
Treated inoc.	6.52	6.47	6.13	6.07	5.78	5.34	5.02	4.76	4.72		

[a] commercial formulation with no additions.

[b] n=3.

[c] inoculated with an equal mixture of *Lb. sakei* and *Lc. mesenteroides* ssp. *mesenteroides* cells to yield 3 log CFU.cm^{-2}.

[d] n=2.