2000

Delaying the spoilage of fresh and processed meats

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Delaying the spoilage of fresh and processed meats

by

Priya Sundaram

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Microbiology

Major Professor: James S. Dickson

Iowa State University

Ames, Iowa

2000
Graduate College
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For the Graduate College
To Mom, Dad, and Amma
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ABSTRACT

Various anti-microbial treatments were studied with respect to their use for the shelf-life extension of meat and meat products. The treatments studied were bacteriocins (pediocin AcH and nisin), organic acids, immobilization, ion chelators, high pressure and irradiation. These were then compared using statistical analysis so as to identify treatment effects on the dynamics of growth of the spoilage bacteria. The experiments focused especially on exploiting the potential of pediocin AcH for this purpose.

Statistical analysis of preliminary data indicated that pediocin AcH had an inhibitory effect on meat microflora although the level of the activity was not adequate for a significant extension in shelf life. Also the effect of pediocin when used in conjunction with immobilization and organic acids was not very clear. Of the organic acids, acetic acid proved more effective in reducing and controlling the numbers of spoilage microflora.

In order to study the use of pediocin AcH, large amounts of the bacteriocin were needed. The first step of the study was therefore geared towards the production of large amounts of the bacteriocin and the analysis of the prepared solution for its level of purity. A scale-up method was developed for the purification of pediocin from 50 liters of culture. Next, the action of the bacteriocin on Listeria monocytogenes, cells in both broth and meat systems was studied. This was done primarily to address the issue of bacteriocin activity in foods systems. Pediocin AcH at 200,000 arbitrary units (AU) effected greater reduction of microbial numbers when acting in combination with 1.5% lactic acid than when used alone. This anti-listerial activity and additive effect were adversely affected by increasing salt concentrations.

In the next step, Pediocin AcH was applied to various samples of ground beef and the surviving microflora isolated and characterized. With low levels of bacteriocin (10,000 AU/g) the survivors consisted most of Staphylococcus aureus, Micrococcus luteus, Pseudomonas spp., Acinetobacter spp., some lactic acid bacteria (LAB) and Bacillus spp., most of which dominate spoilage microflora of meat. Increasing the level of Pediocin to 100,000 AU/g caused a decrease in the numbers of S. aureus, M. luteus, LAB and Bacillus spp. but did not affect Pseudomonas spp. and Acinetobacter spp.

The persistence of Pediocin AcH in raw and cooked meat was studied at different storage temperatures. The bacteriocin persisted in sterile raw ground meat for up to 4 d at 25°C, for 15 d at 7°C and for more than 6 months in frozen samples. In cooked meat, bacteriocin was detectable for up to 6 d when stored at 25°C and for more than 3 months at 4°C. The bacteriocin also remained unaffected by irradiation up to doses of 7.0 kGy and high hydrostatic pressure of up to 100 kpsi.

The next experiment focused on determining if the use of ion chelators (like EDTA and Maltol), Triton X-100, and Tween 80 in conjunction with pediocin extended the target range of the bacteriocin to gram-negative bacteria. The experiment indicated that a combination of pediocin AcH, EDTA, and Triton
X-100 was effective in reducing numbers of *Salmonella typhimurium* DT104 and *Escherichia coli* O157:H7 by approximately 0.5 log units.

In the final experiment, 800,000 AU/ml of pediocin AcH was used in combination with treatments such as 200,000 AU/ml of nisin, 20 mM EDTA, 1% Triton X-100, 1% acetic acid, high pressure at 75,000 psi, vacuum packaging, and irradiation at 3 kGy. Raw meat treated with a combination of the bacteriocins, EDTA, Triton X-100, acetic acid high pressure and vacuum packaging had the lowest bacterial numbers and a shelf life extension of more than 14 d at abuse temperatures of 15°C. Treatment-based differences were very similar among the total aerobic, LAB and Gram-negative populations of the meat samples. In the case of cooked meat, an extension of shelf life of at least 5 d was observed in all samples treated with bacteriocins. Beef cubes treated with a combination of the above-listed biological and chemical treatments with irradiation and vacuum packaging had less than $10^2$ CFU/g of the various populations of bacteria at the end of 27 d of storage at 15°C.

Microbial population data for various treatment combinations in the above experiment were fitted to non-linear regressions. The analysis of these regressions indicated differences in the nature of action of various treatments. Treatments with high pressure caused considerable extensions in the lag phase of microbial growth while irradiation mainly decreased initial numbers and the rate of growth. The use of EDTA and Triton X-100 increased the efficacy of bacteriocins, especially when also combined with high pressure.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

Ever since the dawn of history, mankind has recognized the need to be able to preserve the surplus of food produced. Preservation methods such as fermentation, drying, curing, and cooking have been applied for centuries to extend the shelf life of food (Lück and Jager, 1997). The twentieth century has seen tremendous progress in every aspect of food production, preservation, and distribution. Not only has food production become more efficient and consistent, but also the food is now bought sold and distributed on a global scale. This change in the dynamics of food production and consumption has raised new challenges. Food now has to stay fresh for longer periods of time than before. Producers and processors have to find ways of preserving not only shelf-stable foods (dried vegetables or lentils, etc.) but also perishable ones (fresh meat and dairy products). Also with the increase in volume of food production and distribution to greater numbers of consumers, the risk of food borne disease has also increased. The improved standard of life in most parts of the world has increased life expectancy, but also resulted in a greater percentage of the population susceptible to disease (Madden, 1994).

Meat and meat products are recognized as a major source of zoonotic pathogens. While meat products enter the food chain as sterile muscle tissue, they are mostly free of contaminants and spoilage or pathogenic organisms (Gill, 1992). The slaughtering and processing of the meat introduces varying levels and types of contaminants into the meat and thereby contributes to the eventual spoilage of the product. The traditional methods of preserving meat products include lowering the pH, lowering water activity, cooking, fermentation and curing (Lück and Jager, 1997). In recent years several new spoilage and pathogenic organisms have emerged, in outbreaks most commonly linked to the consumption of meat (Sofos, 1993).

Food borne pathogens are subjected to the same stress conditions in many foods as the barriers in the human intestinal tract (Sheridan and McDowell, 1998). Organisms that have genetically adapted to overcome such stress in food products then stand a better chance of overcoming the same barriers in the human body and causing disease. Food borne pathogens are constantly evolving resistance mechanisms that help them adapt to the stress induced by traditional preservation methods. For example, induction of acid resistance in Escherichia coli O157:H7 also increases its resistance to heating, irradiation and antimicrobials (Buchanan and Doyle, 1997). It therefore becomes necessary not only to improve on traditional preservation techniques, but also to use a series of ‘hurdles’ against the growth of pathogenic organisms in foods and to develop new antimicrobial treatments (Sofos, 1993). The use of bacteriocins and bacteriocinogenic cultures offers one such a new method of selectively targeting undesirable organisms in foods (Hugas, 1998). Also in recent years the use of technologies like irradiation and high hydrostatic pressure treatments have been approved for several foods. The use of organic acids has been expanded from traditional ‘pickling’ and fermentations to sanitation and
decontamination processes. While each preservation technique/treatment can only be improved to a certain extent, and implementation of new technologies is subject to several limitations, combinations of existing methods offer another way to address the issue of food preservation.

Bacteriocins are proteinaceous substances that are antibacterial in nature. They are produced by a variety of organisms. Bacteriocins produced by some Gram-positive bacteria have a broader spectrum of activity. Among Gram-positive organisms that produce bacteriocins, lactic acid bacteria (LAB) are the best studied. Since many species of LAB are used as starter cultures in food fermentations, they show the most promise for use against spoilage and pathogenic organisms (Daeschel, 1989). Pediocin AcH and nisin are two broad-spectrum bacteriocins produced by *Pediococcus acidilactici* and *Lactococcus lactis* respectively (Delves-Broughton, 1990). They are highly inhibitory to several spoilage and pathogenic bacteria. The target spectrum of these bacteriocins is restricted to Gram-positive bacteria. The use of ion-chelators, detergents and other antimicrobial treatments like irradiation, cooking and high hydrostatic pressure has been known to render gram-negative cells susceptible to the action of these bacteriocins.

The objective of this study was to effect shelf life extension of raw beef by a minimum of 15 days and if possible, that of cooked meat by a minimum of 20 days under refrigerated storage (4-7° C). The main focus was on the use of Pediocin AcH and to a lesser extent nisin, as meat preservative agents, and their use in combination with other physical and chemical methods.

Food irradiation and the use of high hydrostatic pressure are highly efficient methods of controlling microorganisms. While irradiation has been approved for use in several products in the United States, high pressure processing is still restricted to the experimental stage. Both methods in themselves do not offer a 'magic bullet' solution to the problems of contamination. Used in combination with other agents, high pressure processing and irradiation are very effective in reducing microbial numbers (Kalchayanand et al., 1998). The aim of this study was also to evaluate the applicability of irradiation and high pressure treatment in combination with other treatments so as to best achieve the above specified shelf-life extension of meat.

Another aspect of this study was extensive statistical analysis of data to identify mechanisms by which various treatments alter the dynamics of microbial growth. This would be an initial step in the development of predictive modeling techniques that can address specific spoilage microflora.

The rationale of the study was to first apply any one of the treatments, determine the shelf-life extension effected and identify drawbacks to the method. The next step was to incorporate other treatments/methods to be used in combination with the first treatment so as to address those specific drawbacks and further extend shelf life. The second step was repeated again until a desired extension of shelf life was achieved. The statistical analysis was used to both identify changes to microbial population dynamics as well as to validate the measurements of shelf life.
Dissertation Organization

This dissertation contains a general introduction, review of literature, a chapter on experimental work, and general conclusions. The literature review is divided into two chapters. Chapter 2 deals with the microbiology of meat and Chapter 3 contains review of relevant literature on the control of spoilage microflora by physical, chemical and biological methods/agents. Chapter 4 is a description of experimental work, the results and discussion. It is divided into one preliminary study and six experiments. Chapter 5 contains a general summary, general discussion and recommendations for future research and is followed by the references. The dissertation uses standard format as prescribed by the thesis office at Iowa State University.
CHAPTER 2. LITERATURE REVIEW: MICROBIOLOGY OF MEAT

Microflora of Meat and Meat Products

Meat and meat products provide an ideal environment for the survival growth of several types of bacteria (Dainty and Mackey, 1992). The growth of any microorganism to high numbers will affect the qualities of meat. In some cases such change is desirable, for example in the production of fermented sausages. However, in most cases the proliferation of microorganisms adversely affects the meat. While spoilage of meat is of great concern from an economic point of view, it is the growth of pathogens that both producers and consumers dread the most. Often, very low numbers of pathogens can render the meat dangerous for consumption. This chapter provides an overview of the different kinds of bacteria that may be found in meat and their role in spoilage, disease and food preservation.

Sources of Contamination

Microbial contamination of meat and meat products can occur from a variety of sources and at several different stages of slaughter and processing (Borch et al., 1996a, Gill, 1979). These include the infection of animals, contamination of meat with feces, hides, feathers, gut contents, etc. during slaughter, as well as from handlers, processing equipment and environment. Among the pathogenic bacteria, Listeria is often introduced into meat from processing environments, Staphylococcus from the skin of the handlers, Salmonella and Escherichia coli from infected animals, and Aeromonas from contaminated water (Smulders, 1995). Spoilage bacteria are also introduced into the meat from the same sources as the pathogens. Hide, fleece or feathers are a major source of spoilage bacteria as they are contaminated with soil and water. Processing steps like scalding and dehairing also select for certain types of bacteria.

Uncooked comminuted meat products generally have higher numbers of both spoilage and pathogenic microorganisms than whole meat (Borch et al., 1996a). The use of lower quality ingredients (e.g. trimmings), the mixing of the wide range of ingredients that spreads contamination, and the increase of surface area resulting from comminution all contribute to increased microbial contamination and growth. Further abuse in the form of improper cooling or temperature abuse, and excessive storage further exacerbates the situation. The addition of other substances like water, spices, etc. in the making of some products also increases the risk of bacterial contamination. Dried spices are often a potential source of spores of Clostridium or Bacillus species. Pathogens most commonly associated with uncooked meat products are Salmonella, E. coli, Campylobacter, Listeria monocytogenes, and Yersinia enterocolitica.

The initial mesophilic bacterial count on meat and cooked meat products is roughly $10^2$ to $10^5$ colony forming unit (CFU)/g (Borch et al., 1996b) and consists of a large variety of species. Only about 10% of these bacteria will be able to grow to high enough numbers so as to cause spoilage. The rate of growth of bacteria in
the meat depends on several intrinsic and extrinsic factors of the meat and the nature of the organism (Dainty and Mackey, 1992).

**Bacterial Attachment**

The first and critical step of contamination is the attachment of bacteria to the surface of meat. This can be differentiated into two stages: the initial reversible attachment, and later the more permanent irreversible attachment (Firstenberg-Eden, 1981). The initial binding depends on several physical factors. The later permanent binding involves the production of extracellular lipopolysaccharides (LPS). Attachment requires relatively short period of time.

The ‘firmness’ of bacterial attachment to a surface is expressed in terms of an ‘S value’ which is used to differentiate between cells in the first reversible stage of attachment, from cells bound irreversibly to the matrix (Firstenberg-Eden, 1981). Motile psychrotrophs like *Pseudomonas* were observed to attach better to meat than non-motile organisms like *Acinetobacter* and *Moraxella* (Farber and Idziak, 1984). There is also a direct correlation between the number of cells in the contaminating material and the number that attach onto the meat.

Other factors that may be involved in bacterial attachment are entrapment by beef tissue fibrils and connective tissue fibers. Bacterial attachment and colonization occurred faster in the presence of calcium chloride than sodium or potassium chloride (Delaquis and McCurdy, 1990). This could be due to the stabilization of the LPS layer of cells which is involved in the second stage of attachment. The relative negative charge on the bacterial cell wall showed a correlation with strength of attachment, but this relation was seen to be greater in lean than fat tissue (Dickson and Koohmaraie, 1989). Some correlation between the relative hydrophobicity of the cell and attachment to fat tissue was observed, based on studies of bacterial adherence to hydrocarbons (Chung et al., 1989). Kim et al. (1996), however, reported that gamma-irradiated and formaldehyde inactivated cells of *Salmonella typhimurium* attached like live cells and differences in surface charge did not seem to affect attachment. The hydrophobicity of live and dead cells was observed to be similar however. *Similarly neither flagella, fimbriae or electrostatic forces were seen to affect bacterial attachment onto poultry skin* (Lillard, 1989), although the presence of these structures may aid attachment by simply providing motility (Firstenberg-Eden, 1981).

**Meat as an Environment for Microbial Growth**

Meat tissue provides a ready source of nutrients for microorganisms. Typically, meat contains about 0.2% glucose and 0.4% amino acids, both of which are the first nutrients to be metabolized by contaminant microflora (Dainty and Mackey, 1992). The pH ranges from 5.6 to 6.5, the lower values being typical of pale soft exudative (PSE) meat and the higher values of dark firm dry (DFD) meat. This range of pH offers a very low selective pressure on contaminating microflora. The higher the pH the less the selective pressure on microflora and the more variety of microorganisms can proliferate in the meat. Adipose tissue has a higher pH
than lean muscle, therefore meat with higher fat content or marbling will spoil more rapidly. Normally the pH of meat drops to values of about 5.4 to 5.8 after storage.

**Spoilage of Meat and Meat Products**

Spoilage patterns depend primarily on the kind of meat product. Meat products may be whole or comminuted and uncooked or cooked. In each case the processing steps vary and so do the sources of contamination and the selective pressure on microflora (Dainty and Mackey, 1992). The conditions under which the meat is stored also determine the type of spoilage microflora that will predominate especially in uncooked products. Refrigeration, and vacuum or modified atmosphere packaging are the two most commonly used methods for restricting the growth of microflora.

**Uncooked Meats**

The shelf life of uncooked meat stored in air, under refrigerated conditions is limited. Off-odors generally appear when numbers reach about $10^7$ CFU/cm² and slime is visible when numbers reach approximately $10^8$ CFU/cm². However, the spoilage patterns vary tremendously with the type of microflora; significantly higher numbers can be present in meat without signs of overt deterioration (Borch et al., 1996a).

Under aerobic storage, spoilage microflora is dominated by gram-negative, psychrotrophic, aerobic rod-shaped bacteria of the genera *Pseudomonas*, *Acinetobacter*, and *Psychrobacter* (Gill., 1986). Of these, *Pseudomonas* spp. are of the greatest importance, as they are highly successful in growing in protein rich foods at low temperatures (Drosinos and Board, 1995). Members of this genus are able to metabolize glucose, a characteristic that gives them a competitive advantage. The most common species found in spoiled meat are *P. fragilis*, *P. lundensis*, and *P. fluorescens*. These produce ethyl esters and sulfur containing compounds, which are responsible for fruity and putrid odors associated with spoilage. *Pseudomonas* spp. often form visible pigmented colonies (Huis-in't-Veld, 1996). *Acinetobacter* and *Psychrobacter* are unable to metabolize glucose and obtain their energy presumably from the oxidation of amino and organic acids. *Brochothrix thermosphacta* is also of importance in aerobically stored meat. This organism flourishes at temperatures above 6°C, and a pH of 6.5. Other bacteria that may also be present in aerobically stored meat are psychrotrophic species of *Micrococcus*, *Staphylococcus* and Enterobacteria such as *Serratia liquefaciens*, *Enterobacter agglomerans* and *Hafnia alvei*. Though present in small numbers, *Enterobacteriaceae* and *B. thermosphacta* influence spoilage patterns by production of branched chain ester residues, acetoin and diacetyl.

In case of uncooked meat stored in vacuum packs, the small amount of residual oxygen (O₂) that may be present is rapidly depleted by respiration. The high carbon dioxide (CO₂) concentrations that result are inhibitory to *Pseudomonas* (Eyles et al., 1993), although they may grow to levels as high as $10^6$ CFU/cm² on residual O₂. Usually, vacuum packaging selectively favors the growth of lactic acid bacteria (LAB) (Leisner et al., 1995). The LAB grow rapidly under conditions of low temperature, low O₂ tensions and high levels of CO₂. They also compete effectively with other microorganisms by virtue of their high affinity for glucose and the
ability to produce a wide range of anti-microbial substances. Predominant LAB in vacuum-packed meat are homofermentative species of *Lactobacillus*, *Carnobacterium*, and *Leuconostoc*. *Lactococcus* also grows although in significantly lower numbers. In vacuum packaged meats stored at higher temperatures and pH, *B. thermosphacta*, psychrotrophic species of *Clostridium*, and Enterobacteria such as *Serratia liquefaciens*, *Shewanella putrefaciens*, and *Providencia* may also grow to significant numbers.

The microflora of meat stored under commercially used modified atmosphere packs (20 to 40% CO₂: 60 to 80% O₂) is very similar to that of meat in vacuum packs. At temperatures below 2°C, the LAB predominate, although here, *Leuconostoc* spp. occur in much higher numbers than in vacuum packs. *B. thermosphacta* is relatively CO₂ tolerant and therefore grows successfully at pH above 5.8. Higher levels of CO₂ inhibit the growth of most gram-negative bacteria (Drosinos and Board, 1995). The presence of O₂ of however permits the growth of *Enterobacteriaceae* and *Pseudomonas* also (Jackson et al., 1991). Meat packed in an atmosphere of 100% CO₂ has a much longer storage life (Greer et al., 1993). The spoilage in this case is almost exclusively lactic acid bacteria especially *Leuconostoc mesenteroides* (Nissen et al., 1996). *B. thermosphacta* may also contribute to spoilage in case of higher pH. The growth of *Pseudomonas* and *Enterobacteriaceae* may also occur (Gill and Harrison, 1989). As in the case of vacuum packaged meat, the most common spoilage pattern is souring. ‘Rancid’ and ‘cheesy’ odors may also appear.

The most common spoilage organism in uncooked ‘fresh’ sausages is *B. thermosphacta* (Gill, 1986). This organism is relatively resistant to sulfites, metabisulphite, which is added to meat to inhibit microbial growth. Spoilage is evidenced by a cheesy or sour odor and taste. Yeast growth may result in binding of the sulfate so as to enable other bacteria like *Enterobacter*, *Citrobacter*, and *Klebsiella* to grow, especially at temperatures above 8°C. The microflora of sausages made without preservatives more closely resembles that of fresh whole meat. *Enterobacteriaceae* predominate at temperatures above 4 to 5°C and *Pseudomonas* at lower temperatures. In case of burgers the spoilage microflora is dominated by species of *Lactobacillus* and *Carnobacterium*. Spoilage is by souring, greening or discoloration and gas formation.

**Cured Meats**

The microbiological stability of cured meats varies with the curing technology. Some products such as bacon, for example, are shelf stable for several months at ambient temperatures (Egan, 1983). Other products require refrigerated storage, and have a shorter shelf life. The pathogen of concern in cured hams, especially dry-cured ones, is *Clostridium* because of its high tolerance to salt (NaCl) and curing agents. In case of spoilage microflora, *Micrococcus* is readily transferred into the meat from pig skin, handling and processing environment. The high NaCl concentration also selects for the proliferation of halophilic species of *Vibrio* especially under conditions of temperature abuse (Huis-in’t-Veld, 1996). Spoilage may also be caused by growth of *Enterobacteriaceae*. In case of prepackaged uncooked cured meats, the use of vacuum or modified atmosphere packaging selects for the predominant growth of lactic acid bacteria (Von Holy et al., 1991).
Cooked Meats

Cooked meats when correctly processed and stored are highly stable products. Microbiological problems occur mostly due to improper control of processing or storage temperatures, or post processing contamination. The initial bacterial levels do not always reflect the shelf life. The conditions of processing and storage play a more important role in the selection of the type of microflora than the initial numbers present in the meat (Nerbrink and Borch, 1993). Even in products cooked in sealed packs, spoilage due to growth of Clostridium and Bacillus species occurs when storage temperatures exceed 7°C. Marginal under-processing may result in the survival of heat resistant strains of Lactobacillus and Enterococcus. Products that are packaged after the cooking stage are more susceptible to bacterial contamination. Spoilage is mostly caused by growth of Lactobacillus and Carnobacterium to levels of $10^3$ to $10^4$ CFU/g and is manifested in production of lactic acid and a sweet-sour odor (Korkeala et al., 1987). In some cases B. thermosphacta may dominate the microflora, giving rise to a characteristic cheesy odor. As in the case of raw meats, the packaging atmosphere also affects the spoilage microflora. The use of air-permeable film allows for the growth of Pseudomonas and other oxidase-positive gram-negative bacteria. Environmental species Enterobacteriaceae including Citrobacter, Enterobacter and Klebsiella are also implicated in the spoilage of cooked meat stored at temperatures above 5°C and under aerobic conditions.

The incidence of Clostridium and some serovars of Salmonella in these products are related directly to whether nitrates were added for color or preservative effect. Of particular concern is the post-processing contamination of cooked products (especially sausages) with L. monocytogenes. This pathogen does not compete well with other bacteria, but flourishes in cooked meat due to the lack of competition and low storage temperature. Staphylococcus aureus, also commonly found in such products, is introduced primarily from human sources.

Fermented Meats

Fermented sausages are comparatively low risk products for the growth of pathogens. This is due to the range of antimicrobial compounds produced by lactic acid bacteria (LAB) used in the fermentation process (Adams and Nicolaides, 1997). The starter cultures tend to control the populations of most contaminants by competitive inhibition. However, two major areas of concern in these products is the growth of S. aureus prior to fermentation and the survival of pathogens like Salmonella and Listeria past the processing stages. The spoilage of fermented meats can occur due to uncontrolled growth of starter cultures. Heterofermentative LAB can also cause off odors and changes in appearance and texture (Hugas, 1998). For example, Leuconostocs that produce only D-lactate can produce an acid off taste in fermented meat products (Montel et al., 1998).
Lactic Acid Bacteria

Introduction

The earliest documented evidence of the use of fermented foods dates back to more than 5,000 years. Most of these fermentations were, and still are effected by lactic acid bacteria (LAB). The concept of LAB as a distinct group of organisms was developed in the early 1900s (Stiles and Holzapfel, 1997). Historically, the LAB have included the genera *Lacticoccus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*. Over the years the relationship between milk souring organisms and other lactic acid producing organisms was recognized. The LAB group has been expanded and some of the species renamed until now the group comprises the following genera: *Carnobacterium*, *Enterococcus*, *Globicatella*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weisella*. This classification of LAB into various genera is based on several factors including morphology, mode of glucose fermentation, growth at different temperatures, configuration of lactic acid produced, range of growth temperatures, range of salt concentrations for growth, tolerance to high and low pH and phylogenetic relationships.

Lactic acid bacteria comprise a diverse group of gram-positive, non-spore forming bacteria, characterized by a fermentative sugar metabolism in which lactic acid is a major end product. They may be either rods or cocci. They are unable to synthesize porphyrin groups and therefore lack catalase and cytochromes. Hence, a majority of these organisms are fermentative, although 'pseudocatalase' may occur in some species. They are chemo-organotrophic and grow only in complex media. The term 'lactic acid bacteria' was initially synonymous with 'milk souring organisms' as most of them were indeed isolated from sour or spoiled milk (Alexsson, 1998).

Lactic acid bacteria occur in diverse food environments, on plants, and also in the genital, intestinal and respiratory tracts of humans and animals. They are often found in habitats rich in nutrients (milk, meat, etc.). They find extensive use in industry in foods such as dairy products, meat, sourdough, vegetables, silage, beverages, etc. Some species are highly pathogenic (e.g., Streptococci) and others are being increasingly used for therapeutic agents. Other uses of LAB being explored are bioconversion of wastes (Martin, 1996), degradation of aflatoxins (El-Nezami and Ahokas, 1998), expression of human and murine interleukins, and in preventive treatment of animal diseases.

Classification

Essentially, LAB can be classified into homolactics and heterolactics based on their carbohydrate metabolism (Egan, 1983). Homolactic metabolism follows the Embden Meyerhof-Parnas (EMP) pathway for glycolysis and results in almost exclusive production of lactic acid. This pathway is used by all LAB except *Leuconostocs*, group III Lactobacilli, Oenococci and Weisellas. Unlike animal muscle, however, LAB may form either D (-) or L (+)-lactic acid, or a racemic mixture of the two isomers. This feature has been used in the identification of some species. Heterolactics utilize either the 6-phosphogluconate/phosphoketolase pathway or
the 'bifidus' pathway of hexose metabolism. This results in the production of significant amounts of other end products such as ethanol, acetate and CO₂ in addition to lactic acid. Several of the LAB can ferment sugars other than glucose, such as mannose, galactose and fructose. These enter the major pathways at the glucose-6-phosphate or fructose-6-phosphate step or, in the case of galactose (in some species), via the tagatose-6-phosphate pathway at the glyceraldehyde-3-phosphate step.

Lactic acid bacteria may be broadly classified into three groups. These groups were originally assigned to lactobacilli and are as follows:

**Group A**: Obligate homofermentative species that ferment hexoses almost exclusively to lactic acid by the EMP pathway. These organisms lack phosphogluconate pathway enzymes and are therefore unable to ferment gluconate or pentoses. This group includes group I *Lactobacilli* and some individual species of other genera.

**Group B**: Facultatively heterofermentative *Lactobacilli*. Hexoses are almost exclusively fermented by the EMP pathway, and the presence of phosphoketolase allows for the fermentation of pentoses as well. This group includes group II *Lactobacilli*, most species of Enterococci, Lactococci, Pediococci, Streptococci, Tetragenococci and Vagococci.

**Group C**: Obligate heterofermentative *Lactobacilli*. Hexoses are fermented by the phosphogluconate pathway to yield lactate, ethanol (or acetic acid) and CO₂ in equimolar quantities. Pentoses may also enter this fermentation pathway. This group includes *Leuconostocs*, group III *Lactobacillus*, *Oenococcus* and *Weisella*.

*Lactobacillus*: These bacteria are gram-positive, strictly fermentative, aero-tolerant or anaerobic, aciduric or acidophilic rods or cocco-bacilli. They can be either homo- or heterofermentative and cannot synthesize porphyrinoids. *Lactobacilli* are found in environments rich in carbohydrates such as mucosal membranes of humans and animals, on plants and in manure. The *L. acidophilus* group, *L. casei* group, and *L. reuteri or L. fermentum* group of *Lactobacilli* are of special importance in the application of LAB as probiotics (Klein et al., 1998).

*Streptococcus*: The streptococci were one of the earliest bacteria to be recognized by microbiologists. This is largely because of their involvement in several human and animal diseases (Stiles and Holzapfel, 1997). Streptococci are split into three genetically distinct groups: *S. sensu stricto, Enterococcus* and *Lactococcus*. The species now classified under *Streptococcus* include pathogenic and oral streptococci. *S. thermophilus* is the only organism classified under this group that is used as a starter culture in foods.

The streptococci are gram-positive, spherical or ovoid cells typically arranged in chains or pairs. The formation of chains is best seen in broth cultures. They are facultatively anaerobic, non-sporing, catalase-negative and homofermentative. Some species produce capsules of extracellular polysaccharide (both glucans
and fructans) when grown in the presence of sucrose. Streptococci have complex nutritional requirements and are usually grown in media enriched with blood, serum or glucose.

**Pediococcus and Tetragenococcus:** The pediococci, by virtue of their association with beer spoilage, were among the first bacteria ever studied (by Louis Pasteur). They are non-motile, non-capsulated, spherical cells 0.36 to 1.43 μm in diameter (Simpson and Taguchi, 1992). They are non-pathogenic, homofermentative, aerotolerant anaerobes with complex nutritional requirements. Pediococci are the only LAB that divide alternatively along two perpendicular axes to form tetrads. They are used in several food fermentations including soy sauce, miso, cheese, sausage, etc. They also cause spoilage of beer, wine and cider. The genus *Tetragenococcus* is more closely related to *Enterococcus* and *Carnobacterium* than *Lactobacillus*.

**Lactococcus:** Lactococci are commonly called ‘mesophilic lactic streptococci’ (Teuber, 1992). Most of the species were originally classified under streptococcus until they were transferred to this group (Schleifer et al., 1985). They are gram-positive, non-motile, coccoid, anaerobic or microaerophilic, and occur in pairs or chains. Some strains produce extracellular polysaccharides. Lactococci are mainly found in association with plant material including grasses, fruit and vegetables. They are usually not found in fecal material or in soil and in very low numbers on cattle. However, the group does include several uncommon species. *L. garvieae* is found associated with mastitis in cows, *L. piscium* from salmonid fish. Most lactococci have been isolated from fermented plant material, milk and milk products.

Lactococci are the most widely used group of the starter culture bacteria. They are the most economically important group and, therefore, have been studied extensively (Teuber et al., 1991). The subspecies *L. lactis* is of special importance in fermented dairy products. Several strains produce bacteriocins of which nisin is widely used as a food preservative (Delves-Broughton et al., 1996).

**Leuconostoc:** Members of the genus *Leuconostoc* are very closely related to lactobacilli and pediococci. These bacteria are asporogenous, non-motile coccoid cells that commonly occur in pairs or short chains (Dellaglio et al., 1992). Leuconostocs are facultative anaerobes with complex nutritional requirements. They produce D (-) lactic acid from glucose. When grown on solid media, the cells are elongated giving them the appearance of short rods. *Leuconostoc* spp. are heterofermentative and are commonly found in plants, dairy products, meat and various fermented products. They are used in the production of fermented foods, wine and dairy products and present predominately in plant products.

**Carnobacterium:** This genus includes gram-positive, catalase-negative, non-sporing, ‘non-aciduric’ rods that are unable to grow on acetate agar at pH 5.6. They have an atypical heterofermentative metabolism, producing L (+) lactic acid, acetic acid, ethanol, formic acid and CO₂.
**Enterococcus**: Enterococci are gram-positive, catalase-negative cocci that produce L-lactic acid homofermentatively from glucose, and also derive energy from degradation of amino acids (Stiles and Holzapfel, 1997). They have a PEP-PTS system for uptake of lactose and other carbohydrates. Enterococci, which do not phenotypically differ from other gram-positive, catalase-negative cocci, are assigned to this genus based on genetic analysis. *E. faecalis* and *E. faecium* are often associated with human and animal infections. Some species of enterococci are used as indicators of fecal contamination in some foods and as starter cultures and probiotics for treatment and prevention of intestinal disorders in humans and animals.

**Starter Cultures**

Starter cultures perform a variety of physiological functions that are vital to the production and preservation of various food products (Hammes and Knauf, 1994; Herrero *et al*., 1996; Hammes and Hertel, 1998; Mayrä-Mäkinen and Bigret, 1998; Montel *et al*., 1998). It must be noted that the very same functions and reactions can cause spoilage if the wrong bacterium is present or if the fermentation process is prolonged beyond the optimum time. These include:

1. **Acid production**: which imparts characteristic flavor, causes lowering of pH which is important in inhibition of spoilage as well as coagulation of proteins. *Lactobacillus sake*, *L. curvatus*, *L. plantarum*, *Pediococcus pentosaceus*, and *P. acidilactici* produce L-lactate and are commonly used in fermented meat products (Montel *et al*., 1998).
2. **Proteolysis**: LAB are only mildly proteolytic compared to other spoilage bacteria. Significant increases in amino acid levels as a result of proteolysis may affect the flavor of a product. The degradation of protein accelerates maturation of the products (Naes *et al*., 1995).
3. **Aroma and flavor formation**: LAB produce several compounds that contribute to the aroma and flavor of food products. These include organic acids, acetaldehyde, diacetyl, acetoin, 2-3 butyleneglycol, dicarboxylns, glyoxal, methylglyoxal, dihydrooxyacetone, etc.
4. **Exopolysaccharide formation**: Exopolysaccharide (EPS) producing strains of LAB are of importance in the production of certain fermented milk products. These strains of bacteria produce EPS as a capsule, closely attached to the cells or loosely attached or excreted slime.
5. **Production of inhibitory compounds**: Apart from pH reduction by acid formation, LAB effect inhibition of competing microflora by production of several compounds targeting such species. These include hydrogen peroxide, CO₂, diacetyl, bacteriocins and secondary reaction products such as hypothiocyanate (Helander *et al*., 1997).
Meat Fermentations

All LAB used in sausage fermentations are homofermentative (Lücke, 1996). The formation of lactic acid from sugars lowers the pH of the meat to below 5.3 and thereby contributes to the inhibition of spoilage and pathogenic bacteria. The LAB also inhibit other species by competition for nutrients and the secretion of several antimicrobial agents. The low pH also helps in the preservation of the product by coagulating soluble meat proteins. Fermentation also results in the accelerated development of curing color due to low pH and the enhancement of flavor and/or of the product by the formation of small amounts of acetic acid, diacetyl, etc. from amino acids. On an industrial scale, the use of LAB is restricted mainly to the production of fermented sausages and to a lesser extent in cooked meat products.

Conditions that favor the growth of LAB during sausage fermentation include a pH below 6.0, water activity in the range of 0.955 to 0.965, and the addition of about 0.3% rapidly fermentable sugar. One hundred to 150 mg of sodium nitrite/kg is added to inhibit clostridia and other competitors. Starter culture species of LAB can be mesophilic, psychrotrophic or thermophilic in nature. *L. plantarum, L. pentosus* as well as several strains of *P. pentosaceus* grow rapidly in sausages at fermentation temperatures of 20 to 25°C. These mesophilic species produce the required amount of lactic acid in a short time thereby ensuring short curing or fermentation periods. The psychrotrophic species include *L. curvatus* and *L. sake*. These species are the most effective at competitive inhibition of other spoilage bacteria at a temperature range of 20 to 22°C but could themselves contribute to spoilage due to their ability to continue producing lactic acid at those temperatures. The thermophilic species of LAB include *P. acidilactici* strains that are used in the manufacture of summer sausages. The fermentation occurs at temperatures of 30 to 38°C and is of very short duration.

Biocontrol by Lactic Acid Bacteria

Lactic acid bacteria have the ability to produce a variety of antimicrobial substances that have an inhibitory effect on a broad spectrum of competing species of microflora. This can be exploited to extend the shelf life of a variety of food products by competitive inhibition. Lactic acid bacteria have been used to inhibit the growth of pathogens in minimally processed vegetables and fruits (Breidt and Fleming, 1997). The production and application of bacteriocins in meat and meat products has been studied extensively (Hugas, 1998).

*L. lactis* subsp. *lactis, L. lactis* subsp. *diacetylicus, L. acidophilus, Streptococcus thermophilus*, are also known to produce antifungal agents that are effective against molds such as *Asperigillus, Penicilium, Rhizopus* and *Cladosporium* (Batish et al., 1997). Roy et al. (1996) isolated a 10.5 kDa protein from cultures of *L. lactis* subsp. *lactis*, which has been associated with inhibition of fungal growth. It has been suggested that the presence of LAB and other competing microflora at levels of 105 to 106 CFU/g in meat is essential to inhibit growth of pathogens and that meats with lower numbers of background microflora have greater potential for spreading disease (Jay, 1996).
Listeria monocytogenes

Listeria monocytogenes was first isolated in 1918 by Dumont and Cotoni, and later identified in 1940 (Paterson, 1940). Murray et al. (1926) first described the organism in 1926 after isolating it from the blood of infected rabbits. They named it Bacterium monocytogenes. The organism was renamed Listerella hepatolytica in 1940, and later changed to Listeria in 1940 (Pirie, 1940). L. monocytogenes was the only known member of this genus until 1961 (Rocourt et al., 1982). Six other species have since been included in the genus, namely: L. innocua, L. ivanovii, L. seeligeri, L. welshimeri, L. murrayi and L. gravi (Rocourt et al., 1982). Of these, only L. monocytogenes and (rarely) L. ivanovii have been known to cause disease in humans. Though L. monocytogenes was recognized as a food borne pathogen in 1929 (Broome, 1993), the first confirmed outbreak of listeriosis was not identified until the 1980s (Schlech et al., 1983). In the next few years several outbreaks were identified as a result of which L. monocytogenes became a major concern in the food industry (Bille, 1990, Bille and Doyle, 1991).

Listeriae are gram-positive, regular, short rods (0.4 to 0.5 \( \mu \)m in diameter by 0.5 to 2 \( \mu \)m in length) with rounded ends. They are aerobic or facultative anaerobic, mesophilic, non-sporeforming and motile by a few peritrichous flagellae at 20 to 25°C. The optimum growth temperature for members of this species is between 30 to 37°C and optimum pH is between 6 and 9. Listeriae are catalase-positive, oxidase-negative, urease-negative, and ferment glucose with the production of L-lactic acid. They are methyl red-positive, Voges Proskauer-negative, hydrolyze esculin and sodium hippurate, do not utilize citrate, and do not produce indole.

Listeria has long been recognized as an animal pathogen. About 90% of animal listeriosis have been attributed to this species. While all strains of L. monocytogenes are potential pathogens, the host immune system is usually effective in neutralizing most infections (Notermans et al., 1998). Most cases of human listeriosis occur in immuno-compromised individuals (Lorber, 1990). These include people with conditions such as neoplastic disease, immuno-suppression, pregnancy, diabetes mellitus, alcoholism, cardiovascular and renal collagen diseases, hemodialysis failure and decreased gastric acidity (Neiman and Lorber, 1980; Ho et al., 1986). Also at risk are neonates, and people of advanced age (over 60). Early symptoms of infection include vomiting, fever, headache and malaise (Farber and Peterkin, 1991). Clinical manifestations include primary bacteremia or infections of the central nervous systems such as meningitis and encephalitis (Schlech, 1996b). It can also cause endocarditis and affect other parts of the body. L. monocytogenes also causes perinatal, neonatal and late-onset neonatal listeriosis, which have a high mortality rate among the infected infants (Schuchat et al., 1991).

Listeriosis occurs both in epidemic as well as sporadic forms (Schuchat et al., 1992). The largest documented outbreak was in Los Angeles County, California in 1985 (Linnan et al., 1988). There were 142 cases and 48 deaths over a period of eight months. A Mexican style cheese was implicated as the vehicle of transmission. Schuchat et al. (1992) estimated the annual incidence of sporadic listeriosis in the United States at 7.4 cases per 1,000,000 population, with a fatality rate of 23%. Most cases of the disease are caused by the 4b
serotype of the pathogen (Schlech. 1996a). Many case studies revealed a connection between listeriosis and the consumption of raw or undercooked foods especially by immuno-compromised individuals. Pinner et al. (1992) conducted a study of food in the refrigerators of 123 patients with sporadic listeriosis. They isolated L. monocytogenes from at least one food product from the refrigerators of 64% of the patients.

L. monocytogenes is considered ubiquitous in the natural environment. Its has been isolated from soil, vegetation, sewage, surface fresh waters, fecal material of diseased and healthy cattle, sheep, goats, pigs and poultry, and even humans. Contamination of food products can therefore occur from either the animal (into meat, milk, etc.) or the environment (into cooked/processed products). L. monocytogenes has been found in a wide variety of foods, especially refrigerated meat and dairy products, several of which have been implicated in cases of listeriosis (Grau and Vanderline, 1992; MacGowan et al., 1994). The organism has been isolated from both raw and processed products including ground meats, fermented sausages, raw meat cuts, packaged wiener, sliced meats and luncheon meats. Among dairy products, L. monocytogenes has been found in raw milk, ice cream and ice milk. Usually, the organism is present in foods in low numbers of less than 100 CFU/g (Doyle, 1994).

The main reason for the success of L. monocytogenes as a food borne pathogen is its ability to grow at refrigeration temperatures. The organism is psychrotrophic growing at temperatures between 3 to 45°C (Wilkins et al., 1972). It is also able to tolerate, under suitable conditions, the high temperature short time pasteurization of milk (Farber and Peterkin, 1991). Also of concern is the halotolerance of the organism. It has the ability to tolerate and grow in 10 to 12% sodium chloride (Billie and Doyle, 1991). Several outbreaks of listeriosis have been traced to dairy and meat products. Nutritionally rich media like meat, and milk increase the resistance of Listeria to antimicrobial agents and treatments (Mackey et al., 1994, 1995). The rise in the number of outbreaks of listeriosis has been linked to the increased use of refrigeration for storage of foods. Being capable of growth at low temperatures, Listeria is able to successfully grow in these foods and compete with organisms that would overwhelm it at higher temperatures. Extended storage at low temperatures also allows for the recovery of sublethally injured cells into viable ones (Mackey et al., 1994).

Control of Listeria in foods mainly targets its heat susceptibility and the fact that the organism is a poor competitor in mixed populations. Food irradiation, the use of high pressure processing, thermal and the use of organic acids and bacteriocins and other chemical agents in cooked products have all shown different levels of success in reducing numbers of this organism in various foods.
CHAPTER 3. LITERATURE REVIEW: THE CONTROL OF SPOILAGE MICROFLORA

This chapter will review literature on the food preservation agents/methods used in this study. The agents/methods are divided into physical methods, namely irradiation and high hydrostatic pressure; biological agents, namely the bacteriocins pediocin AcH and nisin; and chemical agents, namely organic acids. These methods were chosen because they are areas of food preservation technology that have seen the most progress, application or intensive research in recent years.

Physical Methods of Intervention

The past decade has seen extensive study and application of food irradiation as a preservative treatment. The use of irradiation has been approved for several foods and with growing consumer acceptance, the technology is well on its way to being firmly established in the meat industry. In the latter part of this decade the use of high-pressure treatment has also been introduced into the American food industry.

Food Irradiation

The use of radiation to kill microorganisms was conceived in 1896, the same year as the discovery of radioactivity and a year after the discovery of X-rays (Josephson, 1983). The first patents for the use of ionizing radiation to destroy bacteria in foods were issued in 1905. The practical application of this technology was not realized until 1943 when Proctor et al. demonstrated that X-rays could be used to preserve hamburger meat. This long interval between the development of the concept to its practical application was due to the unavailability of sufficient quantities of radioisotopes and machine sources. Food irradiation research began in the United States in the 1950s. In 1958 the Food Additives Amendment to the Federal Food, Drug and Cosmetic Act of 1938 was enacted. This law defines ionizing radiation as a food additive and requires that all new food additives undergo extensive testing. Irradiation is still classified as an additive and is therefore subject to the same regulatory control as additives. The World Health Organization (WHO) has stated that food irradiated with doses of up to 10 kGy is safe for human consumption. Irradiation of pork, poultry, fruits, vegetables, spices and some grains has been approved in the United States thus far.

Food irradiation can be applied to attain a number of objectives such as microbial control, inhibition of sprouting, delay of ripening and senescence, decontamination, insect disinfection and other quality improvements (Tarté, 1996). For the control of microorganisms in foods, different dosage levels can be applied. Radappertization describes the use of ionizing radiation to reduce the numbers of microorganisms to levels below detection limit. This is essentially sterilization by irradiation and takes doses of about 10 to 50 kGy. Radicidation is the treatment of food with doses sufficient to reduce the number of viable specific non-spore-
forming bacteria to levels below detection limit. Dosage levels of 2 to 6.5 kGy can achieve such reduction of most pathogens. Lastly, radurization is the treatment of food with a dose sufficient to enhance the keeping quality of the food by achieving a significant reduction in the numbers of spoilage microflora. Doses of 1 to 5 kGy are sufficient for this purpose.

Radiation can be applied in the form of electron beams, X-rays, or gamma rays. E-beam irradiation subjects the food to a stream of electrons that have been accelerated by a linear accelerator. X-rays are produced when the accelerated electron stream impinges on a steel or tungsten target. Gamma rays are produced by the radioactive decay of radioisotopes. Cobalt-60 ($^{60}$Co) or Cesium-137 ($^{137}$Cs) is used for food irradiation purposes.

The anti-microbial effect of ionizing radiation is caused by a variety of mechanisms (Tarté, 1996). Most of the damage to the cells is done by the production of high energy electrons that are either targeted on the food (e-beam) or produced within it as a result of interactions with X- and gamma rays. These high-energy electrons react with various food molecules (especially water) to produce free radicals that are highly reactive. Free radicals cause extensive damage to cellular components by reacting with them. The damage inflicted on the cell includes deoxyribonucleic acid (DNA) strand breaks, base damage, denaturation of proteins, etc. Any process that restricts the formation of free radicals, their reaction with cellular components or their movement will minimize the damage caused by irradiation. Therefore, freezing, drying or any other form of dehydration, addition of anti-oxidants etc. all tend to yield lower kill reductions of microorganisms with irradiation.

Irradiation has been used to successfully control levels of both pathogens and spoilage bacteria in meat (Green, 1998; Monk et al., 1996; Thayer 1996). Studies have shown significant reduction in numbers of pathogens like S. aureus, C. jejuni, E. coli O157:H7, L. monocytogenes, and Salmonella spp. (Clavero et al., 1994; Chan, 1997).

**High Hydrostatic Pressure (HHP) Treatment**

The origin of high pressure processing of foods may be traced to experiments conducted by I.lite (1899) on milk. A pressure of 667 MPa for 10 minutes at room temperature was seen to bring about a 5 log reduction in the bacterial population of milk. Meat pressurized at 530 MPa for an hour at 52°C was found to stay free of contamination for 3 weeks (Farr, 1990; Lechowich, 1993). In 1914, Bridgman established that high pressure of 709 MPa caused the denaturation of egg white protein. This denaturation is considered to be the primary mode by which high pressure treatment kills microorganisms.

Several subsequent investigations into the effect of high pressure on foods have revealed several advantages to the use of this technology. Pressures of 103 MPa applied to pre-rigor beef for 2 minutes improved the digestibility of meat (Elgasim and Kennick, 1980) without adversely affecting other desirable qualities of the meat like protein efficiency ration, apparent biological value, etc. (Ananth, 1996). Jams and juices treated with high pressure undergo little or no loss of vitamin C and have an extended shelf life. In the United States, cost considerations have been primarily responsible for the restricted use of this technology (Demetrakakes, 1996).
Food companies in the United States have now started using this technology for the preservation of fruit juices, yogurt, guacamole, etc. (Mermelstein, 1997, 1998).

The application of high pressure in food systems is similar to processes used in metallurgy. The food product is sealed in a plastic laminated film like polyvinyl alcohol film and placed in a steel pressure vessel. The vessel is filled with pressure medium (usually water with rust preventive additives) which transmits the pressure to the food package. The pressure is built up in the vessel by a ram-type piston operated by a hydraulic pump (Hori et al., 1992). The metric unit for measurement of pressure is Pascal (Pa). High pressure processing is usually applied in Mega Pascal (MPa) levels, one MPa being $10^8$ Pascals. Other commonly used units are pounds/inch$^2$ (psi) which is equal to $6.8948 \times 10^3$ Pa, and atmosphere (atm) which is equal to $1.01325 \times 10^5$ Pa.

High pressure processing (HPP) affects microbial cells in many different ways depending on several factors like dwell time, pressure level, temperature, nature of organism, etc. The greater the pressure and dwell time, the greater the reduction in numbers of bacteria (Patterson et al., 1995). Gram-positive organisms like Micrococcus luteus, S. aureus, and Streptococcus faecalis are more resistant to HPP than gram-negative organisms such as C. jejuni, Pseudomonas aeruginosa, S. typhimurium, and Y. enterocolitica (Shigehisa et al., 1991). The medium in which the microorganisms are suspended during pressurization is also of critical importance. Enriched media tend to either protect the microorganisms or help in the recovery of sub-lethally injured cells thereby lowering the antimicrobial effect of the treatment (Hoover et al., 1989). Increasing the temperature or lowering the pH of the medium also enhances the anti-microbial activity of HPP as was seen in studies with L. monocytogenes (Stewart et al., 1997). The more rapid the compression and decompression, the more lethal the treatment is to the cells. This is thought to be caused by the injection of gas into cells and the formation and rupture of vacuoles.

The lethal effect of HPP on microorganisms can be due to a variety of biochemical reactions. Volume decreasing processes, like the dissociation of ion pairs, breaking of hydrogen bonds, disruption of hydrophobic interactions, etc. cause reversible and irreversible denaturation of structural proteins and enzymes (Hoover et al., 1989; Hoover, 1993). This in turn affects protein biosynthesis. It is interesting to note that pressurization has been known to cause the enhanced expression of proteins very similar to heat shock proteins in E. coli (Welch et al., 1993). Morphological changes often occur as a result of high pressure treatment. Cells of several organisms become filamentous and with increases in cell length ranging from a factor of 2 to 200. The ribonucleic acid (RNA) content per cell increases but the DNA content per cell remains the same, indicating the inhibition of DNA replication and cell division. The development of pleiomorphic forms has also been reported in some species of bacteria such as Micrococcus aquiviveus (Dring, 1976). A loss of motility is also commonly seen and may be due to restricted supply of energy to the flagellae or the elimination of the structures themselves. Inactivation of spores by high pressure occurs by initiation of germination. It is these germinated vegetative cells that are inactivated by the pressure (Clouston and Wills, 1968; Sale et al., 1970). Another mechanism of pressure inactivation may be increased reactivity of free oxygen radicals (Zobell, 1970).
While HPP by itself may not be highly effective against all types of microflora, it can often be combined with other treatments to provide a synergistic effect. High hydrostatic pressure at 600 MPa by itself did not cause significant inactivation of *Clostridium sporogenes* spores (Mills *et al.*. 1998). However, when combined with heat treatment, about a 3 log reduction in spore numbers was achieved. The same was observed in case of non sporeforming species like *E. coli* O157:H7 and *S. aureus* in milk and poultry (Patterson and Kilpatrick. 1998) and *L. monocytogenes* in fresh pork sausage (Murano *et al.*. 1999). *L. monocytogenes* and *S. typhimurium* cells in fresh pork loin were eliminated by pressurization to 827 MPa for 10 min at 25°C (Ananth *et al.*. 1995). Styles *et al.* (1991) reported that *L. monocytogenes* cells in raw milk had greater pressure sensitivity than those in ultra-high temperature milk. This may be due to the synergistic action of pressure and anti-microbial agents present in raw milk. A combination of high pressure, low pH and bacteriocin was used to inactivate spores of *Bacillus coagulans* (Roberts and Hoover, 1996). A combination of bacteriocins, high pressure and heat was used to achieve significant reductions in the numbers of pathogens like *S. aureus, L. monocytogenes, S. typhimurium,* and *E. coli* O157:H7 (Kalchayanand *et al.*, 1998).

**Biological Agents: The Bacteriocins**

**Introduction**

Many bacteria produce a variety of inhibitory compounds effective against other bacterial species. These include organic acids, hydrogen peroxide, diacetyl, bacteriocins, etc. (Daeschel. 1989). Of these, bacteriocins have the greatest potential for use as food preservatives. The actual role of bacteriocins is still a mystery. They are considered to be part of the arsenal that any bacterial species uses against other competing species in its ecological niche, although this is yet to be demonstrated in a natural system. Scientific literature abounds with detailed reviews on the subject of bacteriocins (Hill, 1995; Jack *et al.*, 1995).

The term ‘Bacteriocin’ was first suggested by Jacob *et al.* (1953) to describe compounds that were produced by certain species against closely related species. In later years the sheer variability of bacteriocins in terms of modes and ranges of action, structure, etc. has made it difficult to precisely define the term. Broadly speaking, bacteriocins are bacterial proteins with anti-microbial activity against (usually) related species. Bacteriocins vary in size, structure, stability, genetic location, post-translational modification, modes of action, composition and range of target organisms. Over the years researchers have used different systems to classify bacteriocins. Class I consists of small, post-translationally modified proteins with a broad host range. These include lantibiotics like nisin, which contain unusual amino acids like lanthionine. Class II consists of small, heat stable unmodified peptides like pediocins that do not undergo any post-translational modifications other than the cleavage of the leader sequence from the pro-bacteriocin molecule. All the members of this group seem to specifically target the cytoplasmic membrane. Class III consists of larger heat labile molecules like Helveticin J.
Anti-microbial Activity

Bacteriocins of LAB share a common mechanism of action. They deplete the Proton Motive Force (PMF) of target cells (Montville and Chen, 1998; Montville and Bruno, 1994). This effect may be energy dependent as in the case of nisin which acts only on energized cells, or energy independent as in the case of pediocin, leuconocin S, etc. (Bruno and Montville, 1993; Montville et al., 1995).

The primary structure of pediocin-like bacteriocins can be roughly divided into two modules: the relatively hydrophilic N terminal region that is well conserved among the various proteins in this group, and the more hydrophobic and diverse C terminal region. Experiments on hybrid bacteriocins indicate that the hydrophobic C terminal region played a crucial role in the determination of anti-bacterial activity (Fimland et al., 1996). Secondary-structure prediction based on amino acid sequence for the N-terminus of pediocin PA-1 call for the presence of two beta-sheets maintained in a hairpin conformation that is stabilized by a disulfide bridge (Chen et al., 1998). This structural model also reveals areas of positively charged residues that indicate electrostatic interactions are probably instrumental in the binding of the bacteriocin to lipid vesicles (Chen et al., 1998).

The pediocin molecule is thought to bind to non-specific receptors on the bacterial cell surface before binding onto specific bacteriocin receptors (Bhunia et al., 1990). Lipoteichoic acid residues might serve as one of the binding sites for the peptide. The addition of pediocin to susceptible cells causes a concomitant efflux of intracellular ions and depletion of cytoplasmic ATP levels (Chen, 1998). This effect is both time and concentration dependent (Chen and Montville, 1995). The loss of ATP may be due to the cell's attempts to maintain the PMF.

The bactericidal effect of nisin is rapid (under 1 min of exposure of sensitive cells). The protein adsorption also seems to involve non-specific receptors and direct interaction between the protein and cell membrane that may be facilitated by the negatively charged cell wall components. Studies in several strains of *L. monocytogenes* cells showed a complete dissipation of the PMF over an external pH range of 5.5 to 7.0 (Bruno et al., 1992). This effect was also observed in spores of *C. sporogenes* PA3679 (Okereke and Montville, 1992). This bacteriocin effects its activity through insertion and pore formation in the cell membrane followed by the rapid efflux of low molecular weight compounds from the cell and depolarization of the membrane (Winkowski et al., 1994). Unlike in the case of pediocin, membrane insertion by nisin requires the target membrane to be charged. Once inserted the protein molecules aggregate to form amphiphilic pores. From a food application point of view, most of the inhibitory action of nisin occurs within the first 30 minutes of addition of the bacteriocin as evidenced by ATP-Bioluminescence assays (Ukuku, 1995).

The lipopolysaccharide (LPS) layer of gram-negative bacteria may provide protection against gram-positive bacteriocins by acting as a barrier between the bacteriocin and the cell membrane. It has been reported that the addition of chelating agents such as ethylenediamine tetra acetic acid (EDTA) that destabilize the LPS layer, tends to make normally resistant gram-negative cells susceptible to the action of nisin. Treatments that adversely affect the cell surface enable the nisin to reach the cell membrane that is its target of action. These
include heat, extended refrigeration and freeze thawing (Mahadeo, 1995). The addition of divalent ions such as magnesium or calcium (that help stabilize the LPS layer) negates the effect on nisin on gram-negative cells such as *S. typhimurium* (Stevens *et al.*, 1992).

**Detection**

For several years methods based on direct or differed antagonism such as spot-on-lawn, cross streaking, lawn overlay, agar-well diffusion and flip-plate techniques were used to detect and screen for bacteriocin production and activity (Muriana and Luchansky, 1993). Nisin activity is usually quantified by the agar diffusion assay. The indicator organism of choice was originally *Micrococcus luteus* ATCC 10420. In the last few years several molecular biology techniques have been developed for this purpose, although the older techniques are still used extensively (Rodriguez *et al.*, 1997; Lewus and Montville, 1991). A modified method was developed to directly detect pediocin AcH in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Bhunia and Johnson, 1992a). The gel was stained for 30 min with Coomassie blue, destained for 1.5 hours and then equilibrated in changing deionized water for 3 hours. The gel was then overlaid with a lawn of indicator bacteria. A zone of inhibition was formed on the indicator lawn around the bacteriocin band.

In spite of the low immunogenicity of bacteriocins, mouse monoclonal antibodies were raised against pediocins RS2 and AcH (Bhunia and Johnson, 1992b; Bhunia, 1994). A dot immunoblot assay developed using these antibodies could successfully detect minimum levels of 32000 arbitrary units. Colony immunoblot assays of the same could successfully differentiate between colonies that were positive and negative for bacteriocin production.

**Pediocin**

Pediocins are produced by bacteria from the genus *Pediococcus*. Several strains of *Pediococcus acidilactici* produce bacteriocins that have a broad range of target organisms. These include pediocins PA-1, AcH (Bhunia *et al.*, 1988), AcM (El Gado *et al.*, 1997), JD (Christensen and Hutkins, 1992), SJ-1, PO2, L50 (Cintas *et al.*, 1995), etc. These bacteriocins differ very slightly in primary structure, target range and other physical and chemical properties. Pediocin AcH and pediocin PA-1 are the best studied of the pediocins, and are produced by *P. acidilactici* strains H and PAC1.0 respectively. Pediocin production in several strains of *Pediococcus acidilactici* has been linked to plasmids (Ray *et al.*, 1989a). The bacteriocin is encoded by a plasmid designated pSMB 74 (Ray *et al.*, 1992).

*Pediococcus acidilactici* can be grown in several simple growth media. Biswas *et al.* (1991) reported high levels of bacteriocin production in TGE broth within 16 to 18h at 30 to 37°C with a final pH of 3.6 to 3.7. The production of bacteriocin was negligible when the medium pH was above 5.0. Pediocin production was seen to occur late during the growth of cells. It was initially thought that the bacteriocin was produced as a secondary metabolite until it was seen that though the pre-pediocin molecules were produced at higher pH, low
pH was required for their conversion to active pediocin AcH (Johnson et al., 1992). Factors influencing the production of pediocin were also studied and optimized by Yang and Ray (1994).

Pediocin exhibits maximum adsorption onto cells at pH 6.0 and minimum at pH 2.0. This property has been exploited in a novel purification method that involves adsorption of the bacteriocin onto producer cells and later elution by simple pH manipulation (Yang et al., 1992).

Pediocin AcH has a broader inhibitory spectrum than most gram-positive bacteriocins. The target organisms include Aeromonas hydrophila, Bacillus cereus, Brochothrix thermosphacta, Clostridium perfringens, Staphylococcus aureus and several Lactobacillus and Leuconostoc species (Bhunia et al., 1988). However, studies showed that sub-lethal injury rendered several gram-negative bacteria like Aeromonas hydrophila, Salmonella typhimurium, Yersinia enterocolitica, Escherichia coli, Pseudomonas fluorescens and P. putida, and some gram-positive bacteria that are otherwise resistant to bacteriocins of LAB, sensitive to pediocin (Kalchayanand et al., 1992). In case of gram-negative cells the LPS layer protects the cell by preventing access of the pediocin into the cell membrane. Sublethal injury or ion chelators disrupt the LPS layer thereby allowing the bacteriocin access to the cell membrane (Schved et al., 1994a, b). Pediocin inhibited most of the organisms responsible for spoilage of vacuum packaged fresh beef (Kalchayanand, 1990). Of these organisms, the most susceptible were the Leuconostoc spp. followed by lactobacilli and Brochothrix thermosphacta. The most resistant organisms were the Pseudomonas spp.

Nisin

There are several detailed reviews on nisin, which amply illustrate that it is the most studied bacteriocin (Delves-Broughton, 1990; Harris et al., 1992b; Hill, 1995; Delves-Broughton et al., 1996). Nisin is produced by several strains of Lactococcus lactis. The bacteriocin is produced as a primary metabolite during the active growth phase of the cells and stops when the culture enters stationary phase (Vuyst and Vandamme, 1992). Active mature nisin is a cationic, hydrophobic molecule with a molecular weight of 3353 and normally exists as stable dimers. It is encoded by the nis A gene (174bp) also termed spaN.

Nisin is the only LAB bacteriocin commercially produced and used. It has been approved for use in foods by the World Health Organization (WHO) since 1968 and the United States Food and Drug Administration (US-FDA) since 1988. Worldwide, nisin is approved for use in food in at least 40 countries. One of the most useful applications of nisin is the prevention of 'late gas blowing' due to the outgrowth of spores of Clostridium butyricum and Clostridium tyrobutyricum. Nisin therefore finds extensive application in processed hard and semi-hard cheeses and cheese spreads. The addition of the bacteriocin allows manufacturers to make a product of higher moisture content without running the risk of spoilage. Nisin is also used in preservation of
both low and high acid canned foods such as vegetables, soups, hams, mushrooms, pasta, etc. The use of bacteriocin in some cases allows for processors to produce foods with higher water activity without compromising the shelf life of the product.

Applications in Food Preservation

Though bacteriocins of several diverse groups of bacteria have been identified and studied, very few have found commercial application. The bacteriocins of LAB have shown the most promise in this area with nisin being at the forefront and the pediocin group not far behind. Pediocin AcH has been successfully used to inhibit *L. monocytogenes* cells in a variety of foods including ground beef, sausage mix, cottage cheese, ice cream and reconstituted dried milk (Motlagh *et al.*, 1992a). Nisin also shows potential for use in controlling malolactic fermentation in wine (Daeschel *et al.*, 1991).

The nature of food product, packaging and microflora are the main determinants of the kind of bacteriocin that is used. One of the main problems in using broad-range bacteriocins as food preservatives are their potential inhibitory activity against both beneficial as well as spoilage microflora especially in fermented food systems. This has been avoided by using bacteriocin-resistant starter cultures in food fermentations where the bacteriocin is added externally or produced by organisms growing in the food (Harris *et al.*, 1992a). Also starter cultures can be genetically engineered to produce bacteriocin endogenously during the fermentation. Narrow range bacteriocins are most suitable for use when the bacteriocin is added to the food with the purpose of inhibiting a specific group of pathogens. In non-fermented products where there is no concern about 'beneficial microflora' broad range bacteriocins can be used.

The application of bacteriocin to food products can be in several forms (Hugas, 1998). They can be added as purified proteins, produced in the food by actively growing cultures or added in immobilized form. Pediocin AcH immobilized on the surface of heat killed producer cells successfully inhibited *L. monocytogenes* on refrigerated chicken meat (Goff *et al.*, 1996). Enhanced control of *L. monocytogenes* cells was achieved by pediocin produced *in situ* in dry fermented sausage (Foegeding *et al.*, 1992). Pediocin PA-1 when applied in dried powder form inhibited *L. monocytogenes* in a variety of foods. The activity was greater in high moisture foods with relatively acid pH than in foods at higher pH and salt concentration (Pucci *et al.*, 1988).

One of the main concerns in the meat industry is post-processing contamination. Organisms like *L. monocytogenes* may be introduced into foods in the stages between cooking and packaging. The organisms then multiply rapidly in an environment lacking any competition and hurdles to growth. Bacteriocins may offer a solution to this problem. Addressing this issue, an experiment was conducted using *in situ* produced pediocins in processed frankfurters (Berry *et al.*, 1991). The treatment was effective in reducing the numbers of *L. monocytogenes* cells for up to 60 days. Packaging films and edible films treated with a formulation of nisin, citric acid, EDTA and Tween 80 effected a 0.4 to 2.1 log reduction in populations of *S. typhimurium* on poultry. A shelf-life extension of 0.6 to 2.2 days was observed when a 3 minute dip in a nisin-containing formulation was followed by wrapping in polyvinylchloride film treated with a nisin formulation (Natarajan, 1997).
Yet another advantage to the use of bacteriocins, especially ones produced by lactic acid bacteria, is the ease at which the gene for bacteriocin can be manipulated. Though most lactic acid bacteria have a GRAS status, not all of them are commercially useful. Commercially used starter cultures can, however, be genetically manipulated so as to render them capable of constitutively producing bacteriocins (Horne et al., 1998; Miller et al., 1998). Construction of bacteriocin producing starter cultures for fermented foods offers a creative solution to the drawbacks of using bacteriocins. This has been achieved using various methods of genetic manipulation including plasmid transfer and conjugal transfer (Kim, 1990; Ray et al., 1989b; Broadbent and Kondo, 1991).

Bacteriocins may also find application as surface decontaminants in the meat industry. Studies on the inhibitory effect of pediocin on the attachment of *L. monocytogenes* to lean beef muscle indicated that the bacteriocin reduced the number of attached bacteria in 2 minutes by 0.5 to 2.2 log cycles (Nielsen et al., 1990). The level of reduction of attached bacteria depended on the levels of bacteriocins.

**Chemical Agents: Organic Acids**

**Introduction**

Organic acids have been used as food preservation agents in a wide variety of foods, including fermented drinks, meats, pickles, canned foods, etc. The acidification of food in order to preserve it from spoilage and to encourage the growth of 'favorable' organisms has been used for centuries. Traditional processes from the making of cheese, yogurt and pickles to the use of vinegar in salads, all function on the same principle. In this respect, acetic and lactic acids have a long history of use in human and animal food. Acetic acid is the main component of vinegar, and lactic acid is found in high levels in yogurt, pickles, etc. The use of organic acids for shelf-life extension of foods has been extensively studied and reviewed (Dickson and Anderson, 1992; Smulders, 1995; Siragusa, 1995). In recent decades, the use of organic acids in the production and preservation of foods has expanded as has the number and types of acids. This section focuses on highlighting some of the major factors that affect the action of these acids and some of the recent developments in their application.

Acids can be introduced into a food in three ways. Firstly, it can be an intrinsic component of the food (e.g., citric acid in citrus fruit). Secondly, the acid can be produced in the food as a result of fermentation (Smulders et al., 1986; Beuchat and Golden, 1989) by certain microorganisms (e.g., starter cultures in dairy products) and lastly the acid may be added to the food (e.g., preservation by addition of vinegar).

Acids may be applied to meat and meat products in different ways. These include immersion, spraying, electrostatic dispersion, fermentation, etc. (Dorsa, 1997; Woolthuis and Smulders, 1985; Smulders et al., 1986). The nature of the meat product and the target microflora are the main determinant of the type of application system.
Anti-microbial Activity

The effectiveness of the acid has been attributed to three factors: the effect of pH, the extent of dissociation of the acid, and the effect specific to the particular acid (Smulders, 1995; Ingram et al., 1956). Opinions are divided on which of the three is the most relevant from the standpoint of anti-microbial action. While some researchers suggest that a reduction in pH is the main reason for inhibition of bacterial growth (Gill and Newton, 1982), others cite the specific nature of the acid to be highly significant (Chung and Goepfert, 1970; Rubin et al., 1982; Eklund, 1983; Buchanan et al., 1993).

Several factors determine the effectiveness of acids in reducing numbers of bacteria in meats. These include:

1. The nature of the meat surface: Reduction in microbial numbers depends on several factors such as the fact content, initial pH, etc. (Dickson, 1991, 1992b; Greer and Dilts, 1995).

2. The nature of the contaminating material: Organic material such as manure tends to interfere with the antimicrobial activity of acids, probably by providing better conditions for attachment and helping buffer the environment of the microflora and added protein moieties for sequestering ions (Dickson and MacNeil, 1991; Dickson and Anderson, 1992).

3. Initial bacterial load: If the initial load of bacterial contamination is low, the use of organic acids offers no significant level of improvement of microbiological quality (Avens et al., 1996; Dickson, 1992a).

4. The concentration of the acid used: In general, the greater the concentration of the acid, the greater its bactericidal activity (Ockerman et al., 1974; Surve et al., 1991). However, the concentration of acid applied to the meat is limited by the effect of the acid on the sensory attributes of the meat. The rate of increase of antimicrobial activity with concentration differs between acids.

5. The type of acid: The efficacy of the acid treatments varies with several factors. Organisms vary in their sensitivity to different acids (Osthold et al., 1983). The use of combinations of acids has been reported to have a greater anti-microbial effect than the use on one type of acid coli (Adams and Hall, 1988). Also the use of buffered acid systems has shown increased anti-microbial activity in studies (Zeitoun and Debevere, 1990).

6. The temperature of the treatment: There are contradicting reports about the effect of temperature on the effect of acids on contaminating microflora. Overall there seems to be a positive relation between the temperature and the effectiveness of organic acids at inhibiting microbial growth (Anderson et al., 1992; Little et al., 1992).

7. The acid sensitivity of the microflora: Acid sensitivities vary greatly with type of microflora. Gram-positive bacteria are generally more acid-resistant than gram-negative bacteria (Huis in't Veld, 1996). There are several intrinsic mechanisms that confer different degrees of acid resistance in bacteria (Russel, 1991). These range from the production of spores, LPS layers, to the enzymes that degrade organic acids. Also some organisms such as Salmonella have a greater ability to develop acid-resistance/adaptance than other bacteria (Dickson and Kunduru, 1995).
8. **The time and duration of application:** The duration of application is an important factor controlling the effectiveness of an organic acid treatment (Prasai et al., 1997; van Netten et al., 1994). In general, the longer the duration of application the greater the decrease in bacterial numbers. For carcass decontamination, a 2-4 log₁₀ reduction can be achieved if organic acids are applied within a matter of hours after slaughter (Mossel 1989).

9. **Post application treatment:** In keeping with the ‘Hurdle’ concept, any subsequent bacteriostatic/cidal treatment of the product enhances the effect of the acids (Leistner and Gorris, 1995). Several groups have successfully extended the shelf life of meat products by combining acid washes with other post application treatments such as modified atmosphere packaging (Zeitoun et al., 1992).

**Salts of Acetic and Lactic Acid**

The salts of acetic and lactic acid are efficient at inhibiting the growth of *Listeria monocytogenes* (Chen and Shelef, 1992). Their use may offer the added advantage of lowering the water activity of the product (Chirife and Ferro-Fontan, 1980), thus supplementing the anti-microbial activity of the acid ions. Salts of acetic and lactic acids have been successfully used to delay the spoilage of products such as fresh pork sausage and beef and poultry products. They have also been shown to inhibit pathogens in these products. Sodium lactate had a greater inhibitory effect on growth of *Staphylococcus aureus* and *Listeria monocytogenes* than sodium chloride (Houtsma et al., 1993). Lactate has been shown to inhibit the same organisms at pH of 7.0 thereby showing potential for use as a preservative for neutral pH foods (Houtsma et al., 1996). Sodium acetate was also effective against *Listeria monocytogenes* when used in combination with EDTA (Golden et al., 1995) and against mesophilic, psychrotrophic, anaerobic and facultative anaerobic microflora on beef when used with potassium sorbates and phosphates (Unda et al., 1990; Mendonca et al., 1989b, 1989a).

**Acids in Combination with Other Treatments**

The use of organic acids in combination with other treatments offers a dual advantage of targeting acid-resistant/adapted organisms as well as increasing the initial reduction of bacterial numbers. Combining acetic and lactic acids washes with hot water washes effected a 5.0-6.0 log reduction in bacterial numbers (Castillo et al., 1998). Acid treatments are also more effective when combined with low water activity as a preservation method for products ranging from diced meat to carcasses (Dickson, 1990).

Other chemical and biological agents that have been successfully used in combination with organic acids are nitrite, transdermal synergists, bacteriocins, spices and spice extracts (Scannell et al., 1997; Syed-Ziauddin et al., 1996; Tamblyn and Conner, 1995).

**Use in Meat Preservation**

The emergence of several new food borne diseases over the past few years has focused attention on the issue of food safety (Doyle, 1994). The use of organic acid washes does not offer a universal panacea for the
contamination problems faced by the meat industry. Indeed they are no substitute for good manufacturing practices (GMPs) that should already be in place in all plants (Snijders, 1988; Snijders et al., 1984). The acid washes of carcasses are by nature an added decontamination step in combination with other treatments (Phebus et al., 1997) to be placed within an HACCP scheme to improve the microbiological quality of product. Currently, acid washes are used extensively in the United States but have not yet been adopted as widely in Europe and other countries. Evidence for the positive effects of use of this technology keeps mounting, and though several questions still remain unanswered, in the course of time, the technology should find application in slaughter and meat processing facilities around the world.

The use of organic acids in foods has found much greater acceptance and wider application (Doores, 1993). Other than the obvious inhibitory effects on several types of bacteria, organic acids can also be used to select for a particular group of organisms from mixed microflora. The incorporation of acids in foods also enhances the anti-microbial action of other treatments such as heating, curing, etc.
CHAPTER 4. DELAYING THE SPOILAGE OF RAW AND PROCESSED MEATS

Introduction

This research project was aimed at the manipulation of microbial populations so as to extend the
shelflife of meat and meat products. Experiments were carried out in several distinct steps each aimed at
addressing various issues dealing with the use of various treatments for this purpose. The first step was a
preliminary study aimed at identifying directions of research and specific information that was needed so as to
develop an effective preservation method. As mentioned in the general introduction (Chapter 1) the
experimental approach involved applying one treatment, evaluating it, identifying drawbacks and addressing
them by combining them with another treatment.

The use of bacteriocin was chosen as the first treatment to be tested for several reasons. In recent years
there has been an increased interest in and demand for naturally occurring food preservatives. Bacteriocins have
all the characteristics that could make them very useful in food preservation (Abee et al., 1995). Bacteriocins
isolated from primarily the lactic acid bacteria group have a relatively broad spectrum of anti-microbial activity
(Marugg, 1991, Barefoot and Nettles, 1993, Tagg et al., 1976). Pediocin, the bacteriocin used in this study, is
produced by Pediococcus acidilactici. Pediococcus acidilactici is a GRAS (Generally Recognized As Safe)
organism, commonly found and used in fermented sausage production. Most pediocins are thermosTable
proteins that function over a wide range of pH. Pediocin AcH has been proven to be effective against both
spoilage and pathogenic organisms including Listeria monocytogenes, Enterococcus faecalis, Staphylococcus
aureus and Clostridium perfringens (Bhunia et al., 1988).

Another reason for the choice of bacteriocin as the first treatment was the fact that nisin, a bacteriocin
similar to pediocin, has already found application in the dairy industry (Delves-Broughton, et al., 1996).
Application in other food systems has not been adopted even though experiments have shown that the
bacteriocin is effective against various spoilage and pathogenic bacteria when used in various meat preservation
and decontamination systems (Cutter and Siragusa, 1998; Scannell et al., 1997; Buncic et al., 1995; El-Khateib
et al., 1993).

Research in the application of bacteriocins (particularly pediocin AcH) for food preservation is
extensive but fragmented, and restricted to very specific areas, e.g., structural characteristics, genetics, mode of
action, etc. (Montville and Chen, 1998; Bukhtiyarova et al., 1994; Chen et al., 1997). Most literature on food
preservation applications focuses on reporting shelf-life extension or initial reduction of bacterial numbers
(Szabo, and Cahill, 1998; Goff et al., 1996; Kalchayanand, 1990) with little or no analysis on the underlying
mechanisms of microbial inhibition or the alteration to spoilage populations or statistical analysis and validation
of the results. Some research focuses on identifying the effect of temperature, pH, fat and protein content on the
activity of bacteriocins in meat (Rogers and Montville, 1994; Degnan and Luchansky, 1992; Pucci et al., 1988).
These studies, however, only report the effect on recoverable activities immediately following addition, and do not test for storage effects. Finally, most studies are conducted with cells in broth systems and are not followed up with tests in food systems where cells can be much more resistant to various treatments (Gaenzle et al., 1999; Parente et al., 1998).

The research on the effect of acids, irradiation and high pressure is much more comprehensive and extensive. The specific effects of these treatments on mixed (spoilage) microflora are well characterized (Dickson and Anderson, 1992; Smulders et al., 1986; Ananth, 1996). Irradiation is approved in foods only in limited doses and therefore cannot be used above certain levels. Also higher levels of radiation tend to adversely affect the sensory qualities of food. High pressure treatment is also limited by equipment design and the fact that several species survive commercial levels of treatment. The use of organic acids in foods is also limited by their effect on sensory attributes of foods. While each of these technologies used alone has limited application, they have different mechanisms of action and different target spectra. Therefore, these treatments were used to address specific drawbacks to bacteriocin application.

**Preliminary Experiment: Shelf-life Extension of Beef Using Various Combinations of Pediocin AcH, Organic Acids and Immobilization**

**Introduction**

The purpose of this preliminary study was to evaluate the effectiveness of bacteriocins in controlling the growth of normal microflora in a meat system. While previous studies have characterized the target spectrum of pediocin AcH, these studies were carried out using pure cultures of various species of bacteria (Bhunia et al., 1988). The specific effect of the protein in a meat system that contains a mixed flora of several strains and species of bacteria in varying numbers had not been examined. This preliminary study focused on the following questions:

1) *Is there an inhibitory action of pediocin AcH on meat surface microflora?*
2) *If so, then how much of a reduction in bacterial numbers is effected?*
3) *How does this reduction in microbial numbers affect the shelf life of the meat?*
4) *What are the drawbacks to the use of pediocin AcH for the purpose of shelf-life extension of meat? What is the reason for these drawbacks? Can these be overcome?*

Another aspect to be explored in this study was the use of organic acids in combination with the bacteriocin. Given the ineffectiveness of pediocin against several gram-negative bacteria, another treatment was needed that could target this group. Organic acids have been used in the preservation of foods for centuries. The most common are acetic and lactic acids, which are extensively used in carcass decontamination (Dickson, 1992, Smulders et al., 1986). Also, gram-negative bacteria are usually more susceptible to the action of these acids than gram positives. Since pediocin AcH is effective against target organisms over a pH range of 2.0 to 7.0
(Bhunia et al., 1988) the use of acids and pediocin may have an added effect (by enhancing the solubility of the bacteriocin) when used in meat.

The use of immobilization was also included in this study for the purpose of identifying other technologies that may be used in combination with bacteriocins so as to enhance its anti-microbial effect. Immobilization in calcium alginate has been a patented process used in the food industry for several years (Kirsop et al., 1989; Lazarus et al., 1976). Studies in meat systems indicate that when immobilized in a calcium alginate gel, acids proved to be even more effective in inhibiting the growth of microorganisms (Siragusa et al. 1992).

Materials and Methods

Culture and Growth Conditions

*Pediococcus acidilactici* strain H was used for the production of pediocin AcH. The strain was maintained on slants of MRS lactobacillus agar (Difco) supplemented with 2% yeast extract. *L monocytogenes* Scott A was used as an indicator to assay the pediocin AcH and was maintained on Tryptic soy Agar (Difco) slants and was subcultured every two weeks.

Preparation and Assay of Pediocin AcH

*Pediococcus acidilactici* strain H was grown in MRS Lactobacillus broth supplemented with 2% yeast extract for 16 h. The culture was then placed in a 75°C water bath for 15 minutes in order to kill the cells and denature proteolytic enzymes, and then centrifuged at 5000g for 12 minutes. The cell free supernatant was used as crude pediocin.

0.1 ml of an 18 hr culture of *L monocytogenes* Scott A was used to inoculate 10ml of Tryptic Soy broth supplemented with 0.6% yeast extract (TSBYE) and the culture grown for 8 hours at 37°C. TSBYE with 0.75% agar was prepared and part of this ‘soft’ agar was used to prepare ‘bottom’ agar plates (containing 10ml in each plate). The remaining ‘soft agar was held at 40-42°C to be used as ‘top’ agar. Indicator cells of about 10^6 CFU were mixed with 5ml of this ‘top’ agar and overlaid onto the ‘bottom’ agar plates and allowed to cool. The crude pediocin was diluted in a two-fold series and 5μl of each dilution placed on the surface of the indicator lawn and then incubated at 37°C. The inhibitory strength was expressed in arbitrary units (AU). One AU was defined as the inverse of the highest dilution that produced an inhibition zone of diameter greater than 2mm on the indicator lawn (Tagg and McGiven, 1971).

Treatment of Meat

Lean beefsteak (about 10% intramuscular fat) was cut into 1x1x2 cm cubes. The cubes were washed in sterile peptone water before being subjected to the various treatments. Five cubes received each treatment. The organic acids were applied at concentrations of 1% (vol/vol) and the pediocin at 10^6 AU. The various treatments included are summarized in Table 4.1:
Table 4.1. Treatments used and Abbreviations used to denote them

<table>
<thead>
<tr>
<th>Non-Immobilized Treatments</th>
<th>Immobilized Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Immobilized control</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>Immobilization + Acetic acid</td>
</tr>
<tr>
<td>Pediocin + Acetic acid</td>
<td>Immobilization + Pediocin + Acetic acid</td>
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<td>Pediocin</td>
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<tr>
<td>Pediocin + Lactic acid</td>
<td>Immobilization + Pediocin + Lactic acid</td>
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<td>Lactic acid</td>
<td>Immobilization + Lactic acid</td>
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After treatment the cubes were shaken free of excess liquid, placed in petridishes, sealed and stored at 4-7°C.

Immobilization in Alginate

The meat samples were dipped in a sterile 2% (wt/vol.) solution of sodium alginate until evenly coated. They were then dipped in sterile 1M calcium chloride solution for 10 min at room temperature to cross link the alginate. In case of combined treatments, the acids and the bacteriocin were incorporated into the calcium chloride solution before the alginate coated meat pieces were dipped into them.

Estimation of Microbial Growth

Meat samples were stored at 4-7°C to simulate refrigerated storage. Sampling was carried out at weekly intervals. A cube was removed from the petridish, placed in a filtered stomacher bag, diluted ten-fold (wt/wt) with peptone water and stomached for 2 min. The resulting filtered suspension was then serially diluted and plated onto Tryptic Soy Agar (TSA) and MRS lactobacillus Agar, to determine the numbers of the total aerobic microflora and the lactic acid bacteria respectively. The plates were incubated for 48 h (at 37°C for TSA, 30°C for MRS) before counting. Microbial numbers were converted to and recorded as log₁₀ CFU/g.

Statistical Analysis

The data were analyzed using Statistical Analysis System (SAS). While microbial populations were expected to follow the typical sigmoid growth pattern, the populations that were in the exponential growth phase over the period of study would exhibit almost linear relationships between time and log values of microbial numbers. Linear regressions were fitted for each treatment so as to obtain an approximated idea of the differences between the treatments in terms of initial reduction in microbial numbers, rate of growth, degree of extension of shelf life as well as growth pattern. The intercept of each line on the Y-axis gives the microbial numbers at time zero. Thus, subtracting the Y-intercept value of each treatment from the corresponding value of the control gives the initial reduction in numbers for that treatment. The slope of each regression line gives the average rate of growth over the period of the study. The time at which the microbial numbers reached a level of
$10^8$ CFU/g was taken as an indicator of shelf life. The more closely a population follows exponential growth over the period of the study, the better the fit of the linear regression model. An estimate of how well the model fits the data is given by the R-square value. As the R-square value approaches 1.00, the fit of the model is better.

In order to compare growth patterns of two treatments, the t-value of the two treatments was calculated using the following equation:

$$t = \frac{(A) - (B)}{\sqrt{\frac{a^2}{4} + \frac{b^2}{4}}}$$

where A and B are the parameter estimates (slope or Y-intercept) of the two treatments and a and b are the corresponding standard errors. Using normal approximation, if $t \geq 2$ then we reject the null hypothesis ($H_0: A = B$) and conclude that the parameters of the two treatments are significantly different. If $t < 2$ we do not reject $H_0$ and conclude that the two treatments are not significantly different.

**Results and Discussion**

Tables 4.2 and 4.3 contain the results of the statistical analysis of the data for total aerobic microflora (Tables 4.2a and b) and lactic acid bacteria (Tables 4.3a and b). These can be used in conjunction with the plots of the average observed values (Figs. 4.1-4.7) to analyze the dynamics of microbial growth with different treatments. The Tables contain estimates of the slopes and Y-intercepts of the linear regressions fitted to data from the various treatments, as well as the corresponding standard errors (in parentheses). Also given are values of 'approximate estimates of spoilage time'. These are calculated estimates of when the corresponding curves reach log population values of 8.0 in Figs. 4.1-4.6. This is taken as a rough estimate of time for spoilage. 'N' refers to the number of repetitions of the entire experiment.

**Effect of Organic Acids**

Both acetic and lactic acids produced significant reductions in microbial numbers. In case of lactic acid application, the microflora (specifically the lactic acid bacteria) recovered and re-established their populations to spoilage levels (Figs. 4.1-4.6). Table 4.1 lists the abbreviations used to denote the various treatments discussed in this section. Though the initial reduction of microbial numbers was approximately 2.0 log units, both the estimated and average observed times for spoilage (at $10^8$CFU/g) were not greatly extended as compared to acetic acid treatments. This may be because lactic acid bacteria are the main spoilage microflora in the meat. Being producers of lactic acid, they may have a higher resistance to it than to acetic acid. This explanation is further strengthened by the fact that all the spoiled meat samples had the distinct 'sour' odor of spoilage by LAB. Further characterization of the resistant microflora would be needed to confirm this. It is also possible that other organisms that are resistant to lactic acid (but not lactic acid bacteria) could have survived this treatment and proliferated to spoilage levels at refrigeration temperatures. Gram-positive spore formers such as *Clostridium*, and *Bacillus* are in this category. These, however, cause a distinct 'rotten' or 'putrefactive' odor in the meat which was not discerned in the spoiled samples. Acetic acid was much more effective in controlling the growth of bacteria in the meat effecting a considerable extension in shelf life. The microflora of acetic acid
Table 4.2. The observed and estimated parameters of the growth curves of total aerobic microflora

**a. From various non-immobilized treatments**

| Trt. | N | Observed Y intercept Log (CFU)/g | Approx. Spoilage time (Week) * | R-square value | Estimate of Y-Intercept Log (CFU)/g | $P > |T|$ | Estimate of Slope in Log (CFU/g/wk) | $P > |T|$ | Estimate of Spoilage time (Week) |
|------|---|--------------------------------|-------------------------------|----------------|------------------------------------|------|-----------------------------------|------|-------------------------------|
| C    | 4 | 4.44                           | 1.65                          | 0.67           | 4.69 (0.65)                        | 0.0001 | 1.97 (0.39)                      | 0.0003 | 1.68                          |
| L    | 4 | 3.58                           | 3.9                           | 0.79           | 2.65 (0.41)                        | 0.0001 | 1.38 (0.18)                      | 0.0001 | 3.88                          |
| PL   | 4 | 3.29                           | 3.95                          | 0.76           | 2.40 (0.45)                        | 0.0001 | 1.52 (0.22)                      | 0.0001 | 3.68                          |
| P    | 4 | 3.49                           | 2.9                           | 0.50           | 4.10 (0.63)                        | 0.0001 | 1.12 (0.28)                      | 0.0011 | 3.48                          |
| PA   | 4 | 3.36                           | NA                            | 0.18           | 2.59 (0.37)                        | 0.0001 | 0.30 (0.15)                      | 0.0608 | 18.0                          |
| A    | 4 | 3.35                           | NA                            | 0.03           | 2.96 (0.36)                        | 0.0001 | 0.11 (0.15)                      | 0.4768 | 45.8                          |

**b.: From various immobilized treatments**

| Trt. | N | Observed Y intercept Log (CFU)/g | Approx. Spoilage time (Week) * | R-square value | Estimate of Y-Intercept Log (CFU)/g | $P > |T|$ | Estimate of Slope in Log (CFU/g/wk) | $P > |T|$ | Estimate of Spoilage time (Week) |
|------|---|--------------------------------|-------------------------------|----------------|------------------------------------|------|-----------------------------------|------|-------------------------------|
| IC   | 4 | 3.67                           | 1.75                          | 0.74           | 4.15 (0.57)                        | 0.0001 | 2.0 (0.35)                       | 0.0001 | 1.93                          |
| IL   | 4 | 2.96                           | 4.25                          | 0.83           | 2.87 (0.34)                        | 0.0001 | 1.39 (0.16)                      | 0.0001 | 3.69                          |
| IPL  | 4 | 3.37                           | 4.25                          | 0.81           | 3.06 (0.33)                        | 0.0001 | 1.18 (0.14)                      | 0.0001 | 4.19                          |
| IP   | 4 | 4.39                           | NA                            | 0.39           | 4.77 (0.44)                        | 0.0001 | 0.63 (0.20)                      | 0.0056 | 5.1                           |
| IPA  | 4 | 3.62                           | NA                            | 0.09           | 3.44 (0.25)                        | 0.0001 | 0.14 (0.1)                       | 0.1993 | 5.2                           |
| IA   | 4 | 3.68                           | NA                            | 0.006          | 3.21 (0.31)                        | 0.0001 | 0.04 (0.13)                      | 0.7457 | 119.8                         |

**Note:** The listed values are of total microflora with values for standard error in parentheses. N refers to the number of repetitions of each treatment (Trt).

* In some cases the curves do not cross or attain values close to $X=8.0$ log units, therefore, estimates of spoilage time were not made (NA).
Table 4.3. The observed and estimated parameters of the growth curves of lactic acid bacteria

<table>
<thead>
<tr>
<th>Trt</th>
<th>N</th>
<th>Observed Y intercept Log (CFU)/g</th>
<th>Approx. Spoilage time (Week)</th>
<th>R-square value</th>
<th>Estimate of Y-Intercept Log (CFU)/g</th>
<th>P &gt;</th>
<th>T</th>
<th>Estimate of Slope in Log (CFU/g)/wk</th>
<th>P &gt;</th>
<th>T</th>
<th>Estimate of Spoilage time (Week)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>4</td>
<td>3.31</td>
<td>1.65</td>
<td>0.54</td>
<td>3.76 (0.57)</td>
<td>0.0001</td>
<td></td>
<td>1.29 (0.34)</td>
<td>0.0028</td>
<td>3.29</td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>4</td>
<td>2.84</td>
<td>3.8</td>
<td>0.84</td>
<td>2.15 (0.38)</td>
<td>0.0001</td>
<td></td>
<td>1.55 (0.16)</td>
<td>0.0001</td>
<td>3.77</td>
<td></td>
</tr>
<tr>
<td>PL</td>
<td>4</td>
<td>1.87</td>
<td>3.6</td>
<td>0.91</td>
<td>1.42 (0.32)</td>
<td>0.0005</td>
<td></td>
<td>1.90 (0.16)</td>
<td>0.0001</td>
<td>3.46</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>4</td>
<td>2.95</td>
<td>NA</td>
<td>0.51</td>
<td>3.32 (0.58)</td>
<td>0.0001</td>
<td></td>
<td>1.07 (0.26)</td>
<td>0.0008</td>
<td>4.37</td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>4</td>
<td>2.20</td>
<td>NA</td>
<td>0.28</td>
<td>1.91 (0.31)</td>
<td>0.0001</td>
<td></td>
<td>0.33 (0.13)</td>
<td>0.0194</td>
<td>18.75</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>4</td>
<td>2.05</td>
<td>NA</td>
<td>0.4</td>
<td>1.86 (0.24)</td>
<td>0.0001</td>
<td></td>
<td>0.34 (0.10)</td>
<td>0.0026</td>
<td>18.05</td>
<td></td>
</tr>
<tr>
<td>IC</td>
<td>4</td>
<td>2.39</td>
<td>NA</td>
<td>0.5</td>
<td>3.56 (0.79)</td>
<td>0.0009</td>
<td></td>
<td>1.56 (0.50)</td>
<td>0.0085</td>
<td>2.85</td>
<td></td>
</tr>
<tr>
<td>IL</td>
<td>4</td>
<td>2.23</td>
<td>4.0</td>
<td>0.85</td>
<td>2.37 (0.39)</td>
<td>0.0001</td>
<td></td>
<td>1.58 (0.17)</td>
<td>0.0001</td>
<td>3.56</td>
<td></td>
</tr>
<tr>
<td>IPL</td>
<td>4</td>
<td>1.99</td>
<td>4.5</td>
<td>0.81</td>
<td>1.97 (0.39)</td>
<td>0.0001</td>
<td></td>
<td>1.45 (0.17)</td>
<td>0.0001</td>
<td>4.15</td>
<td></td>
</tr>
<tr>
<td>IP</td>
<td>4</td>
<td>3.24</td>
<td>NA</td>
<td>0.44</td>
<td>3.99 (0.48)</td>
<td>0.0001</td>
<td></td>
<td>0.77 (0.22)</td>
<td>0.0026</td>
<td>5.22</td>
<td></td>
</tr>
<tr>
<td>IPA</td>
<td>4</td>
<td>2.95</td>
<td>NA</td>
<td>0.005</td>
<td>2.63 (0.19)</td>
<td>0.0001</td>
<td></td>
<td>0.02 (0.08)</td>
<td>0.7652</td>
<td>268.5</td>
<td></td>
</tr>
<tr>
<td>IA</td>
<td>4</td>
<td>3.44</td>
<td>NA</td>
<td>0.007</td>
<td>2.80 (0.33)</td>
<td>0.0001</td>
<td></td>
<td>0.11 (0.14)</td>
<td>0.4337</td>
<td>72.5</td>
<td></td>
</tr>
</tbody>
</table>

Note: The listed values are of LAB with values for standard deviation in parentheses. N refers to the number of repetitions of each treatment (Trt)

* In some cases the curves do not cross or attain values close to X= 8.0 log units, therefore, estimates of spoilage time were not made (NA).
Fig. 4.1. Non-Immobilized Treatments (TSA)

Fig. 4.2. Immobilized Treatments (TSA)
Fig. 4.3. Non-Immobilized Treatments (MRS)  
('m' refers to microbial counts of lactic acid bacteria on MRS agar)

Fig. 4.4. Non-Immobilized Treatments (MRS)  
('m' refers to microbial counts of lactic acid bacteria on MRS agar)
Fig. 4.5. Effect of immobilization

Fig. 4.6. Comparison of the effect of treatments on total aerobic microflora and lactic acid bacteria

('m' refers to microbial counts of lactic acid bacteria on MRS agar)
treated meat did not follow the typical exponential growth of log phase. The bacterial numbers decreased in the first week and did not reach 4-log units/g during the course of the study (values in bold in Tables 4.2a and b and 4.3a and b). Since the estimated slope of these curves were very close to zero, the R-square value was very low. However, Figs. 4.1-4.4 indicate that microbial spoilage did not occur even at the end of 4 weeks. This is equivalent to a shelf-life extension of at least 2 weeks.

The linear regression model is a local approximation of the true non-linear relationship between cell numbers and time, and it cannot be used to make ‘out of sample predictions’ beyond 1-2 time periods (weeks). In case of acetic acid treatments, calculated estimates of spoilage time reach much more than 2 weeks and therefore are not reliable. In case of other treatments, the estimates of spoilage time fall closer to 1-2 sampling time periods and closely follow the observed values.

**Effect of Immobilization**

In all cases, the numbers of lactic acid bacteria (LAB), though smaller, followed the total microbial counts closely, except in cases of treatment with acetic acid and pediocin. Comparisons of non-immobilized treatments with the corresponding immobilized ones were carried out using the t-test described earlier. The results are listed in Table 4.4. Table 4.1 lists the treatments corresponding to the abbreviations used in this section. Significant effects of immobilization are seen in the case of treatments with acid and pediocin combinations (values in bold font). However, the process of immobilization results in greater number of survivors but slower growth rates. Thus the resulting extension of shelf life is not great in case of treatment with lactic acid. In case of acetic acid, the estimated shelf life is too unreliable to determine if immobilization has a significant effect. In case of use treatment with pediocin alone, there is a significant decrease in the growth rate, which gives an estimated 0.85 week increase in shelf life. This still does not compare with shelf-life extension effected by treatments with acetic acid. The plotted growth curves of average values (Fig. 4.5) indicate that immobilization caused an observed extension of shelf life, but not by more than 0.1-0.6 weeks.

The exact reason for the increased initial survival of cells in immobilized treatments is unknown. The calcium ions used to stabilize the alginate gel may have a protective effect on the cells (for example, stabilizing the LPS layer of gram-negative cells). It is also possible that the increase in water content caused by the gel may actually encourage bacterial growth, and increase the diffusion of proteolytic enzymes from the meat surface causing the degradation of the pediocin or the moisture at the surface to remain higher negating the effects of dehydration. Further experiments are needed to determine the precise reason for this.

Lastly, immobilization significantly reduced both the initial number and the growth rate of lactic acid bacteria in meat treated with acetic acid alone. Interestingly these effects were not significant in case of the total microflora. This may be because other surviving bacteria such as enterobacteriaceae may have grown more rapidly and contributed to higher numbers of the total microflora. This can be further tested by plating for enterobacteriaceae on a selective medium such as Violet Red Bile Glucose (VRBG) agar. This step is to be included in subsequent experiments.
Table 4.4. Effect of immobilization on the total aerobic microflora (TSA) and lactic acid bacteria (MRS) of various treatments

<table>
<thead>
<tr>
<th>Treatments Compared</th>
<th>Calculated t for parameter</th>
<th>Treatments Compared</th>
<th>Calculated t for parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial #s</td>
<td>Slope</td>
<td></td>
</tr>
<tr>
<td>C Vs IC (TSA)</td>
<td>1.20</td>
<td>-0.12</td>
<td>C Vs IC (MRS)</td>
</tr>
<tr>
<td>L Vs IL (TSA)</td>
<td>-0.83</td>
<td>-0.83</td>
<td>L Vs IL (MRS)</td>
</tr>
<tr>
<td>PL Vs IPL (TSA)</td>
<td>-2.37</td>
<td>2.61</td>
<td>PL Vs IPL (MRS)</td>
</tr>
<tr>
<td>P Vs IP (TSA)</td>
<td>-1.74</td>
<td>2.85</td>
<td>P Vs IP (MRS)</td>
</tr>
<tr>
<td>PA Vs IPA (TSA)</td>
<td>-3.81</td>
<td>4.88</td>
<td>PA Vs IPA (MRS)</td>
</tr>
<tr>
<td>A Vs IA (TSA)</td>
<td>-1.05</td>
<td>0.71</td>
<td>A Vs IA (MRS)</td>
</tr>
</tbody>
</table>

Effect of Pediocin

The pediocin was very effective in reducing microflora initially. The difference in TSA and MRS counts suggested that a majority of the affected microflora were lactic acid bacteria. However, with time, the remaining bacteria successfully grew back to levels comparable to control as indicated by the decreasing difference in the TSA and MRS counts, especially after week 2 (Fig. 4.6). This illustrates one of the major limitations to the use of bacteriocins. A minimum number of bacteriocins have to bind to the surface of a cell before any inhibitory activity can take place. Once bound to a cell, the bacteriocin molecules are unavailable for action on other cells. Therefore, unless initial levels of bacteriocin are high enough to bind to all susceptible cells, some sensitive cells will survive the treatment and grow unchecked in the food. Also of interest is the fact that a combination of pediocin and lactic acid (PL) produced a much greater initial reduction in lactic acid bacteria than application of lactic acid alone (L) (Fig. 4.7). This effect is not seen in the total aerobic microflora. The decrease in the initial numbers of bacteria is, however, accompanied with a significant increase in growth rate of the survivors. Thus the increase in shelf life is only 0.3 weeks. In case of acetic acid application, this combined effect on the lactic acid bacteria is not seen. By the end of week 1, the lactic acid bacteria populations of all pediocin treatments begin to constitute most of the total microflora.

The lack of the expected combined effect between acids and bacteriocin may have been due to a number of factors. The use of unpurified pediocin perhaps is the main reason, as it contains high levels of salt, and the pediocin may be in the form of inactive aggregates in the presence of added acids. The increase in ionic strength caused by the acids themselves cannot be ruled out. Another factor to consider is the use of culture supernatant as a source of the bacteriocin. The supernatant contains spent MRS medium that may therefore encourage the growth of spoilage microflora.

The cells that survive bacteriocin treatment alter the dynamics of growth of spoilage microflora, but from the above results it is clear that this change in microflora does not translate to a significant extension in shelf life. This population of cells grows rapidly and to high numbers. Cells that survive bacteriocin application...
Fig. 4.7. Effect of pediocin and acid on total microflora and lactic acid bacteria

('m' refers to microbial counts of lactic acid bacteria on MRS agar)
may be resistant gram-negative bacteria, resistant gram-positive bacteria, or susceptible gram-positive bacteria that have survived pediocin treatment. Bacteria that are susceptible to pediocin may have survived because the added levels of bacteriocin were not high enough or because the bacteriocin was degraded by the proteolytic enzymes in the meat thereby decreasing the impact of its application.

Conclusions

The results of the experiments clearly indicate that pediocin does have an inhibitory effect on the normal microflora of beef. The levels of bacteriocin used in this study effected a reduction of almost 2 log units of normal surface microflora. However, this initial reduction did not result in the control of spoilage populations later during storage. This may be due to several reasons, one of which is the use of crude culture supernatant as a source of pediocin. Also the levels of applied pediocin may not have been high enough to target all the susceptible cells. It is difficult to estimate the exact levels of bacteriocin required to kill all the susceptible cells in a meat sample simply because of the tremendous variation in the microflora from sample to sample. Conversely, the initially applied levels of pediocin may have been greatly reduced by proteolytic activity in the meat (Bhunia et al., 1988; Holzapfel, 1995). An estimate of the shelf life of the bacteriocin in the meat is needed in order to determine the duration of its action against target bacteria. Surviving bacteria need to be characterized in order to determine what further steps can be taken to eliminate them, or control their numbers.

Another limitation to the use of bacteriocin for the shelf-life extension of beef has been the ineffectiveness of the protein against gram-negative bacteria (Bhunia et al., 1988). Other methods may need to be applied either to directly target gram-negative cells, and/or to render those bacteria susceptible to the bacteriocin. The use of organic acids for this purpose gave mixed results. While acetic and lactic acids by themselves were effective in reducing initial levels of bacteria, and in case of the former, controlling growth of spoilage bacteria during storage, they did not exhibit any increased activity when used in conjunction with bacteriocins. This also illustrates the necessity for testing antimicrobials in food systems as studies in broth systems indicate a definite combined effect of bacteriocin and acid (Janes, 1999). This again may be traced to several factors including protective effect of meat, the use of crude preparation of pediocin, etc. The effect of using a purer form of bacteriocin on meat microflora needs to be examined.

The use of immobilization of both acid and pediocin on the surface of meat did not produce an appreciable extension of shelf life of the meat as compared to other treatments. Other methods of targeting resistant cells may need to be studied. These include new preservation techniques such as irradiation and high hydrostatic pressure.

The statistical analysis of the data provided valuable information about the nature of microbial populations and their growth dynamics. Lactic acid bacteria were highly susceptible to treatments with pediocin, acids and combinations of the same, but this did not always result in lower numbers of total aerobic microflora. However, a linear growth model proved to be inadequate for the purpose of detailed analysis. A non-linear regression may be better applied to growth curves of this nature. In order for a non-linear regression to be fitted
to these growth curves, more data are required of the population numbers in all phases of growth. Therefore, further experiments require the populations to be followed over longer periods of time and sampled more frequently over the period of growth.

While pediocin and other bacteriocins have great potential for use in food preservation, considerable study is needed in the areas of food safety applications before systems can be developed that fully utilize this potential. Of particular importance is the study of interactions between bacteriocins and various food components and how these interactions affect the anti-microbial activity of the protein. The great variations in the composition and the numbers of normal meat microflora and the variations in the composition of the meat cubes (size, shape, fat content, etc) introduce a high degree of error to the data. This makes the statistical analysis and validation of the results all the more crucial. Also the characterization of the changes effected by bacteriocins on the nature of the bacterial population in the food will provide valuable information for the design of anti-microbial treatments using bacteriocins.

**Experiment 1: Production and Partial Purification of Pediocin AcH**

**Introduction**

Preliminary experiments on the nature of the action of pediocin AcH on the normal surface microflora of beef indicated that the bacteriocin effected an initial reduction of the same. A crude form of pediocin was prepared by growing producer cells in MRS broth enriched with yeast extract, then removing the cells by centrifugation. The other components of the supernatant (proteins, salts, etc.) may have interfered with the action of the bacteriocin, or affected the growth of the surviving cells in the treated meat. To address this issue, this experiment focused on the production of large quantities of semi-purified pediocin AcH that could be used for further experiments. The resulting preparation was tested for purity.

**Large-Scale Production of Pediocin**

The experiments described in this dissertation required the production of large amounts of pediocin AcH. While most established protocols were for the production of small volumes of upto 1.0 liter, there were none published for larger volumes of recovery (Bhunia et al., 1988). Therefore, a method to scale up the production and purification process was needed. The following method was developed to produce larger amounts of bacteriocin and was based on the purification method as described by Yang et al. (1992). Modifications for scaling up of the process were incorporated wherever needed. The fermentations were carried out as follows at the Iowa State University Fermentation Facility. The steps followed were:

1. **Trypticase Glucose Yeast Extract (TGE) broth** was prepared by adding the required amount of dry constituents (1% Glucose, 1% Yeast extract, 1% Trypticase, 0.2% Tween 80) to a 50 liter fermentor, re-
hydrating it with 50 liters of distilled water. The broth was sterilized in the fermentor and allowed to cool to 35°C.

2. A 12 hour culture of *Pediococcus acidilactici* was grown in 500 ml of MRS broth with 2% yeast extract at 35°C and inoculated into the fermentor. The fermentation was carried out at 35°C for 18 hours, with a minimum of stirring (required to keep temperature control).

3. At the end of 18 hours, the fermentation was stopped by raising the temperature of the culture to 75°C over a period of 25 min, and cooling it down to 20°C. This step inactivates most proteases in the medium that would otherwise degrade the bacteriocins, while having little or no effect on the bacteriocin itself. Stirring rate was increased to facilitate heating and cooling. *(At this point, 2 liters of the culture were removed. 1 liter was mixed with an equal volume of cold 95% ethanol, stirred for 10 hours, and the resulting precipitate collected by centrifugation. The 70% ammonium sulfate was added to the other 1 liter of culture supernatant, and the mixture stirred for 10 hours at 4°C. This precipitate was also collected by centrifugation. Both preparations were then freeze dried and stored until analysis.)*

4. The pH of the culture was adjusted to 6.0 by the addition of 0.5M sodium hydroxide. At this pH pediocin exhibits maximum adsorption onto the cell surface. The culture was then stirred for 1-1.5 hours (cells from previous fermentations were added to the culture at this point).

5. The culture was drained from the fermentor and filtered using a Micon hollow fiber cartridge (0.22 microns). A 1ml sample of the filtrate was collected and the rest of it discarded. The filtration was continued until less than 3 liters of concentrated cell suspension remained.

6. Six liters of 5 mM sodium phosphate (pH 6.5) was added to the cells suspension, which was again re-filtered until less than 2 liters of cell suspension remained. To minimize loss of cells in the cartridge, the cell suspension was then transferred to centrifuge bottles and centrifuged at 15000 x g for a minimum of 10 min. Then 1ml of the supernatant was collected as well as the cell pellet. The rest of the supernatant was discarded.

7. The cell pellet was re-suspended in 6 liters of 100 mM sodium chloride at pH 2.0 as the adsorption of pediocin onto cells decreases with pH and the bacteriocin itself is adversely affected by pH values lower than 2.0. For maximum desorption of the bacteriocin from the cells, the suspension was stirred at 4°C for 5 to 6 h. centrifuged, and the pH of the supernatant readjusted to 6.0 with sodium hydroxide.
8. This semi-purified pediocin was then filter sterilized, a small sample collected for assay and the rest divided into 500ml volumes and frozen until use.

9. The samples of precipitates (from step 3), filtrate (from step 5), supernatant (from step 6), and semi-purified pediocin were assayed for activity using the spot-on-lawn method and *Listeria monocytogenes* Scott A as an indicator as described in the preliminary study.

10. When assayed for activity the final pediocin solution had about 1,600,000 AU/ml of activity. The culture supernatant had about 400,000 AU/ml of activity, and the washings about 800-1600 AU/ml, giving a yield of about 40% of pediocin activity. The yield was calculated using the following formula:

\[
\text{Pediocin activity of final preparation} \times \text{Volume of final preparation} \times 100
\]

\[
\text{Pediocin activity of culture supernatant} \times \text{Volume of culture supernatant}
\]

Giving \((1,600,000 \times 6) \times 100 = 41.4\%\)

\((400,000 \times 58)\)

The ethanol precipitate had an activity of about 400,000 AU/ml and the ammonium sulfate precipitate contained 200,000 AU/ml of activity. It must be noted here that the high levels of salt in the solution may inhibit the activity of the ammonium sulfate precipitate of pediocin.

**Analysis of Semi-purified Pediocin AcH by SDS-PAGE**

The semi-purified pediocin AcH prepared as outlined above was analyzed to determine its degree of purity. These experiments were performed at the University of Arkansas-Fayetteville under the guidance of the Dr. Michael Johnson and his research group.

1. The semi-purified pediocin and culture supernatant were both freeze dried, re-suspended in a small volume of sterile distilled water and filter sterilized. The samples of culture supernatant precipitates from Step 3 (as described in the previous section) were also treated in the same way.

2. All samples were then dialyzed for 24 hours against sterile distilled water using Spectrapor 6 dialysis membrane (MWCO 1,000 Da). The water was changed every 6-8 hours and the dialysis carried out at 4°C.

3. At the end of dialysis, both samples contained a fine precipitate of pediocin. This is attributed to the formation of aggregates of pediocin at very low ionic strength (due to its hydrophobic nature). The precipitate was collected by centrifugation and re-suspended in de-ionized water.
4. These samples were then analyzed by SDS-PAGE as described by Schagger and Von Jagow (1987). The proteins were run on a 12% polyacrylamide gel for approximately 5 hours. A Tris-Tricine cathode buffer was used.

5. The gels were then stained (with 0.1% Coomassie Blue) for 30 min, photographed, and de-stained with acetic acid and methanol for 1.5 hours. The de-staining solution was changed every 30 min (Bhunia and Johnson, 1992). The gels were then further washed in distilled water for 3 hours. Fig. 4.9 shows the gels prepared by this method. A 50% ethanol precipitate and a 70% ammonium sulfate precipitate of the culture supernatant were also analyzed.

6. The gels which were then washed in distilled water for 3 hours, placed on a petridish containing MRS soft agar (MRS broth + 0.75% agar) and overlaid with MRS soft agar containing about 10^6 cells of Lactobacillus plantarum. Lactobacillus plantarum was chosen as an indicator due to its ability to form a dense lawn of cells in MRS agar that allows for better visualization of inhibition zones. The MRS agar was used so as to minimize the growth of contaminants by selective inhibition. The plates were allowed to set and incubated at 30°C for 2 days.

7. The bacteriocin from the gel bands diffused out of the polyacrylamide and into the MRS agar. This resulted in a clear zone of inhibition around the specific band of protein (Figs. 4.10 and 4.11).

8. The purified pediocin sample exhibited one band of molecular weight around 2.6 kDa, and two faint bands of about 5.0 kDa (Fig. 4.8). The former clearly exhibited inhibitory activity and was taken to be pediocin AcH (Figs. 4.9 and 4.10).

The two larger proteins exhibited no inhibitory activity and their function is unknown. They may be inactive precursors of pediocin or proteins that bind to the bacteriocin so as to be purified with it. Further tests are needed in order to identify them. These proteins were not seen when a second gel was run using 30 hour culture supernatant and pediocin purified from it, which suggests that they may be precursors of the bacteriocin itself.

**Conclusion**

The scale-up fermentation method was successful in producing large volumes of semi-purified pediocin AcH. The final preparation had fewer proteins in solution than either the culture supernatant or preparations using ammonium sulfate or ethanol, with pediocin AcH as the predominant protein. The agar overlay test clearly indicated the presence of the pediocin band in both culture supernatant as well as semi-purified pediocin and confirmed the inhibitory activity of the protein. In terms of activity, this preparation had 4-8 fold higher activity.
Fig. 4.8. SDS-PAGE gels of various preparations of Pediocin AcH

(For Gel A culture was grown for 30 hours while for Gel B the culture was grown for 18 hours. Note that the bands of unknown protein seen in Gel B are not present in Gel A. The other two lanes in Gel B are other preparations of Pediocin. Lane E contained a precipitate of culture supernatant with 50% cold ethanol and Lane A contained a precipitate of culture supernatant with 70% ammonium sulfate.)
Fig. 4.9. Gel with agar overlay, as viewed from the top of the petridish

Fig. 4.10. Gel with agar overlay as viewed from the bottom of the petridish
(as measured by AU) against \textit{Listeria monocytogenes} as the indicator than the other preparations. Other methods of purification tested (ethanol and ammonium sulfate precipitation) yielded solutions with several protein bands and much lower activity.

In order for any preservative treatment to find commercial use, it has to be cost effective (Holzapfel, 1995). In the case of any biological or chemical agent, the ease of production and purification of the compound determine the cost of treatment. The main producers of broad-range bacteriocins have so far been LAB, which are generally recognized as safe (GRAS) organisms that can be grown on relatively inexpensive substrates or media. For example, high yields of nisin and pediocin PO2 were obtained in a continuous culture fermentation using an immobilized-cell, packed-bed bioreactor and a whey permeate medium supplemented with lactose, yeast extract, Tween and other components (Liu, 1997).

Commercially used preparations of bacteriocins tend to contain several impurities, as purification to higher levels is not cost-effective. Commercially used nisin preparations contain high levels of sodium chloride, and milk/ whey proteins. These preparations are effective in the preservation of several types of food products such as processed hard and semi-hard cheeses and cheese spreads (Delves-Broughton \textit{et al.}, 1996). The same applies for pediocin application. Whey permeate medium supplemented with 1% yeast extract and 0.1% Tween 80 and at an initial pH of 6.5 was used to grow \textit{P. acidilactici} and produce bacteriocins (Liao, 1992). The bacteriocin containing whey permeate medium was dried to powder form and added to pasteurized milk where it showed inhibitory activity against \textit{L. monocytogenes}.

As seen in the preliminary experiments, the use of crude preparations of bacteriocins can limit effectiveness. Methods such as ammonium sulfate precipitation, chromatography, reverse phase HPLC, etc. are commonly used for the purification of bacteriocins from culture media (Carolissen-Mackay \textit{et al.}, 1997). These methods are not cost effective for the production of large quantities of bacteriocin for commercial purposes. In recent years, methods involving adsorption of the bacteriocins onto diatomaceous earth, salicylic acid, and rice hull ash, have also been developed as cost effective methods of commercial production of semi-purified bacteriocins (Janes \textit{et al.}, 1998; Coventry \textit{et al.}, 1996). These methods, however, involve the use of one or several non-food grade compounds such as sodium dodecyl sulfate (SDS) for the recovery of bacteriocin from the adsorbent material. The above-described method uses simple food-grade solutions for purification. The resulting preparation has fewer impurities than other methods (such as ammonium sulfate or ethanol precipitation), and fewer than in food grade preparations of nisin currently in use.

Though the use of producer cells as adsorbent is the simplest method of purification, there were no reports of this method being used for production of large volumes of bacteriocin (Yang \textit{et al.}, 1992). The above described scale-up method substitutes the use centrifugation with hollow fiber filtration thereby adapting the method for commercial scale preparations. Thus it was concluded that this method of production and purification (pH based) of pediocin was best for use in subsequent experiments.
Experiment 2: The Effect of Organic Acid and Salt on the Anti-listerial Activity of Pediocin AcH in Beef

Introduction

Before bacteriocins can be successfully formulated into food preservatives, there is a need to achieve a thorough understanding of how they are affected by various food components such as enzymes, salt, fat and protein, and other parameters such as temperature and pH. Previous studies have shown that pediocin AcH is active at a pH range of 2.0 to 7.0, and binds to a greater extent to protein than fat (Degnan and Luchansky, 1992; Yang et al., 1992). It is degraded by proteolytic enzymes but is resistant to temperatures of up to 121°C (Bhunia et al., 1988). The last part of this experiment was a test of the effectiveness of pediocin AcH against *Listeria monocytogenes* in meat, and how it is affected by the presence of salt. As mentioned earlier, the research on pediocin action in foods is fragmented and rarely are studies conducted in 'retail quality' food systems. Though studies have been conducted on the combined action of acid and pediocin, they have been tested in broth or agar media and not in meat. It is the aim of this study to compare not only the action of pediocin and acid but also the effectiveness of these treatments when applied to bacteria attached to meat.

Preliminary experiments on the use of pediocin AcH for the shelf-life extension of meat were carried out using culture supernatant as a crude form of pediocin. Results of these experiments indicated that there was no great difference in the reduction/control of microbial numbers caused by the application of organic acids alone and acids in combination with pediocin. Part of the reason for this lack of an additive effect between acid and bacteriocin may be the use of culture supernatant as a crude form of pediocin. In this experiment, the anti-microbial effect of acid alone and the combination of acid and semi-purified pediocin was studied to see if such an additive effect occurred with the use of a less impure form of the protein.

*Listeria monocytogenes* was chosen as the target organism in this study for two reasons. Firstly, the organism is highly susceptible to pediocin AcH thereby making it a good indicator, and secondly it is a major pathogen of concern in several meat and meat-based products. In recent years there have been numerous outbreaks of foodborne listeriosis in the United States and abroad (Bille, 1990; Schlech, 1996; Notermans et al., 1998). The main foods implicated in these outbreaks are meat and dairy products. The risk of food contamination with *Listeria* spp. is considerable mainly because of the ubiquitous presence of the organism. Though it is relatively susceptible to heat processing, the main concern is of *Listeria monocytogenes* occurring as a post-processing contaminant in many processed and ready-to-eat foods. Several studies have demonstrated the anti-listerial activity of pediocin AcH when applied as protein or produced in meat as a part of the fermentation process (Kalchayanand, 1990; Berry et al., 1991; Motlagh et al., 1992).

In the first part of this experiment, broth cultures of *L. monocytogenes* were tested with combinations of pediocin with different levels of lactic acid. The results of this test were visually examined to see if there was a better reduction in microbial numbers when the two agents were used together as compared to when they were
used alone. It also was used to roughly estimate how high an acid concentration (used alone or in combination) was needed to kill $10^7$ cells of *L. monocytogenes*, i.e., at what concentration of acid there were no survivors seen growing on the recovery media. In the second part of the experiment, this study was repeated on *L. monocytogenes* attached to meat. The main issue addressed by the second part of the experiment was if there was an increase in the survival of the pathogen when it was attached to beef tissue and if any effect observed in the broth test still occurred.

**Materials and Methods**

**Broth Test for Inhibition**

In order to confirm the inhibitory effect of pediocin and organic acid on *L. monocytogenes*, cells were first tested in broth. A 12-h culture of *L. monocytogenes* was grown in TSBYE at 37°C. Then $10^6$ cells were transferred to 1 ml of pediocin in a sterile micro centrifuge tube containing 200,000 AU of activity, and concentrated lactic acid (85%) was added to give the appropriate percentage. The cells were vortexed briefly, incubated for 10 min at room temperature, and 0.1 ml of the suspension spread-plated, in duplicate, onto TSAYE plates. The plates were incubated at 37°C for 4 d and visually scored for inhibition. Lactic (0.0, 0.5, 1.0 and 1.5%) was tested alone or in conjunction with pediocin AcH. The plates were compared and scanned.

**Inoculation and Treatment of Meat**

Lean beefsteak was cut into cubes (1 cm x 2 cm x 1 cm) and irradiated at 10 kGy. The beef cubes were stored on ice until use. They were then washed in sterile distilled water and inoculated by a 20-min immersion in a 12 hour culture of *L. monocytogenes* diluted to $10^6$ CFU/ml with 0.1% peptone water at room temperature. After inoculation, the cubes were immersed in 100 ml of their respective treatment solutions and shaken gently for 10 min at room temperature.

**Enumeration of Survivors**

After treatment, the cubes were placed in stomacher bags and diluted twofold (wt/wt) with 1% peptone water (a two-fold dilution). The samples were stomached for 2 min and the resulting homogenate serially diluted as needed and plated on TSAYE. The plates were incubated at 37°C for 4 d.

**Effect of Pediocin and Acid**

Inoculated beef was immersed in treatments containing 0, 0.5, 1.0, 1.5 and 2.0% lactic acid alone and in combination with 200,000 AU/ml of pediocin AcH. The bacteriocin was thawed and the treatments prepared about 20 minutes before the addition of the beef. To test the effect of salt concentration on the activity of pediocin and acid, the inoculated beef was immersed in treatments containing 0.1, 0.5 and 1.0 M sodium chloride alone and in combination with 200,000 AU/ml of pediocin AcH and/or 1.5% lactic acid. Two to three
cubes of beef (depending on the weight) were used per experiment for each test. Both of these experiments were performed thrice each.

**Statistical Analysis**

The results of the first part of the experiment were only visually analyzed for a rough estimate of the presence and absence of a difference between treatments. Results from the second and third parts of the experiment were analyzed by using the Statistical Analysis System to see if the difference between treatments were statistically significant.

Assuming a linear relation between the concentration of acid and the number of survivors, the data for the second part of the experiment were fitted to the following equation:

\[ y = \beta_0 + \beta_1 \text{acid} + \beta_2 d + \beta_3 d \cdot \text{acid} + e \]  

where \( d = 0 \) for treatments with acid alone and \( d = 1 \) if treatments involved a combination of pediocin and acid and \( e \) is the error term. \( \beta_0 \) is the intercept value (when no treatment is applied), \( \beta_1 \) is the effect on the intercept of adding acid, \( \beta_2 \) is the effect of pediocin alone on the slope of the curve and \( \beta_3 \) is the effect on the slope of the curve of pediocin and acid. The estimates of \( y \) therefore would be

\[ E(y | d = 0) = \beta_0 + \beta_1 \text{acid} + e \]  

\[ E(y | d = 1) = \beta_0 + \beta_1 \text{acid} + \beta_2 + \beta_3 \text{acid} + e = (\beta_0 + \beta_2) + (\beta_1 + \beta_3) \text{acid} + e \]  

Substituting \( a_0 \) for \( (\beta_0 + \beta_2) \) and \( a_1 \) for \( (\beta_1 + \beta_3) \) gives:

\[ E(y | d = 1) = a_0 + a_1 \text{acid} + e. \]

The first step is to test whether acid has an effect on the number of survivors by testing the hypothesis \( H_0: \beta_0 = 0 \) and \( H_0: \beta_1 = 0 \) using a standard two-sided test. The next step is to examine the effect of adding acid on the number of survivors by testing the hypothesis \( H_0: \beta_2 = 0 \). The final step is to examine whether adding pediocin also produces a change in the slope of the curve with the hypothesis \( H_0: \beta_3 = 0 \) vs. \( H_0: \beta_3 \neq 0 \) and can be carried out using a t-test. The test of the hypotheses that \( \beta_0, \beta_1, \beta_2, \) and \( \beta_3 \) are significant reveals that in this experiment, acid and bacteriocin act alone and in combination.

For part 3 of the experiments the significance of the effect of salt on the number of survivors of acid treatment (A), pediocin treatment (P), and Pediocin + Acid treatment (PA) was tested. The data were also analyzed (using a general linear model procedure) with regards to the differences between survivor numbers of treatments PA and P to see if this changed with salt level.

**Results and Discussion**

**Combined Effect of Pediocin and Acid**

Preliminary tests of the action of pediocin and acid on a suspension of *L. monocytogenes* in peptone water showed a distinct combined effect between the action of bacteriocin and lactic acid. At levels of 1.5% acid in combination with pediocin, there were no detectable survivors on the plates. This is in contrast with the results seen in the preliminary study where a combination of acid and bacteriocin had no greater inhibitory
activity than that of the acid alone. This suggests again that the combined effect seen may be from the use of a semi-purified pediocin and/or the test of effect in peptone water instead of meat. It is from these preliminary data that the acid concentrations were set for the next two experiments. The specific reason for the observed effect between acid and bacteriocin remains unknown. The lowering of pH may help keep the bacteriocin in solution (synergism), and acids may also attack the cell membranes thus causing the cells to be even more susceptible to the bacteriocin or each treatment may kill cells that have been sub-lethally injured by the other (additive effect).

When tested on beef, 200,000 AU/ml of pediocin effected a 4.0 log reduction of L. monocytogenes on beef. The average reduction in microbial numbers is plotted in Fig. 4.11 ('P' denotes treatments with pediocin alone and 'PA' with pediocin and acid). Even 2% lactic acid did not achieve more than a 2.0 log reduction under the same conditions. A combination of pediocin and lactic acid was much more effective against the organisms. A combination of 200,000 AU with 1.5% acid reduced Listeria by 5.3 log units. When 2.0% acid was used, the number of survivors was below detection level. There is a significant difference in the acid sensitivity of bacteria based on their attachment to the meat. Studies on poultry showed that planktonic salmonellae were significantly more sensitive to the action of organic acids than sessile cells (Tamblyn and Conner, 1995). Therefore, it was important to both establish that the inhibitory effect occurred on cells, and that this effect also occurred, although to a lesser extent, in meat systems. The buffering activity of the meat may be responsible for the increased acid resistance of L. monocytogenes attached to meat. This reinforces the need for testing all treatments and treatment combinations in actual food systems.

The results of the statistical analysis are tabulated in Table 4.5, which gives the estimated values of $\beta_0$, $\beta_1$, $\beta_2$ and $\beta_3$. The $F$ value of the model was 1925.352 with a Prob $> F$ value of 0.0001 and an R-square value of 0.9955, indicating a significant treatment effect and that the model fit the data well. As can be seen in the Table, the values of $\beta_0$, $\beta_1$, $\beta_2$, and $\beta_3$ are significant. Significant values of $\beta_0$ and $\beta_1$ indicate that there is an effect of adding acid on the number of survivors $\beta_0$ and this effect is denoted by $\beta_1$. A significant value of $\beta_2$ indicates that adding pediocin alone also affects the number of survivors and $\beta_3$ indicates the effect pediocin has on the relationship between the number of survivors and acid concentration, i.e., $\beta_1$ or the slope. Negative value of $\beta_1$ indicates that as acid concentration increases, number of survivors decreases. The same effect occurs with the addition of pediocin as indicated by the negative value of $\beta_2$.

Table 4.5. Results of statistical analysis of part 2

| Model Parameter                                    | Estimated Value | Standard Error | Prob $>|T|$ |
|----------------------------------------------------|-----------------|----------------|------------|
| $\beta_0$ (initial level of cells) Log (CFU/g)      | 7.2251          | 0.0771         | 0.00001    |
| $\beta_1$ acid effect on intercept                 | -0.4977         | 0.0611         | 0.0001     |
| $\beta_2$ pediocin effect on intercept             | -6.1297         | 0.1327         | 0.0001     |
| $\beta_3$ acid and pediocin effect on slope        | 0.6881          | 0.0599         | 0.0001     |
Fig. 4.11. Effect of pediocin at different acid concentrations

Fig. 4.12. Effect of Salt concentration on pediocin and acid activity
Effect of Salt Concentration

The application of 200,000 AU of pediocin with 1.5% acid at 0.1, 0.5 and 1.0 M sodium chloride also showed the same pattern of inhibition as in the previous experiment as indicated by Fig. 4.12 (‘A’ denotes treatment with acid alone. There was a distinct combined effect between the acid and bacteriocin. Pediocin alone at 0.1 M sodium chloride produced about 3.0 log reduction in L. monocytogenes. The number of survivors increased with salt concentration. At 1.0 M sodium chloride there was only a 2.0 log reduction. At low salt concentration, the combination of lactic acid and pediocin resulted in 0.9 log lower number of survivors than with pediocin alone. However, this difference in inhibition was 0.4 log units at 0.5 M sodium chloride. At 1.0 M sodium chloride concentration, there were more survivors in beef treated with a combination of pediocin and acid. Several minute colonies appeared on the plates after 72 h of incubation. These were confirmed to be Listeria cells by plating again on modified Oxford (MOX) medium.

The statistical analysis of the results indicates a definite influence of salt levels on both the effects of acid alone and the combination of pediocin and acid. The results of the analysis are summarized in Table 4.6. The R-square value for the model was 0.997638. The above data show that there is a significant effect of salt on the number of survivors. Also there were significant differences in the number of survivors based on treatment.

The contrast C1 shows that the difference in (P-PA) at 0.1M salt and (P-PA) at 1.0 M salt is significant. Also, contrast C2 indicates the average of (P-PA) at these levels is not significantly different from (P-PA) at 0.5M salt indicating that this value falls somewhere midway between the other levels. This confirms a definite inhibitory effect of salt concentration on the difference in microbial reduction caused by acid alone and

<table>
<thead>
<tr>
<th>Salt Level</th>
<th>N (repetitions)</th>
<th>Mean</th>
<th>Standard Deviation</th>
<th>Treatment</th>
<th>N (repetitions)</th>
<th>Mean</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 mM</td>
<td>9</td>
<td>4.541111</td>
<td>1.1073893</td>
<td>A</td>
<td>9</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>9</td>
<td>3.738889</td>
<td>1.7431397</td>
<td>P</td>
<td>9</td>
<td>3.332222</td>
<td>0.2573314</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>9</td>
<td>4.075556</td>
<td>1.4549751</td>
<td>PA</td>
<td>9</td>
<td>3.023333</td>
<td>0.8082852</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees of Freedom</th>
<th>F value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>8</td>
<td>950.51</td>
<td>0.0001</td>
</tr>
<tr>
<td>Salt</td>
<td>2</td>
<td>206.24</td>
<td>0.0001</td>
</tr>
<tr>
<td>Treatment (P, A, PA)</td>
<td>2</td>
<td>3404.58</td>
<td>0.0001</td>
</tr>
<tr>
<td>Salt × Treatment</td>
<td>4</td>
<td>95.60</td>
<td>0.0001</td>
</tr>
<tr>
<td>CI</td>
<td>1</td>
<td>173.52</td>
<td>0.0001</td>
</tr>
<tr>
<td>C2</td>
<td>1</td>
<td>2.64</td>
<td>0.1217</td>
</tr>
</tbody>
</table>
a combination of acid and pediocin. Salt level not only adversely affects the anti-microbial action of pediocin and salt but also the additive effect of the two treatments.

The decreased efficacy of pediocin AcH with increased salt concentration may be due to the hydrophobicity of the protein. In case of nisin, increased salt increases the ionic strength of the solution thereby altering the conformation and solubility of the protein (Liu and Hansen, 1990; Hurst, 1981). The same may happen in case of pediocin. At very high concentrations of salt the addition of acid may further force the pediocin to alter its conformation or form aggregates. This may explain why the pediocin activity is more when used alone than in combination with acid at 1.0 M sodium chloride. Increased ionic strength may also affect the binding of pediocin to cell surface receptors.

Conclusions

This experiment shows that a combined effect between the action of pediocin and acid occurs in both broth and meat systems. Previous studies indicated that the combination of acid and bacteriocin had a combined effect on target organisms, but these studies were not carried out in meat systems, but in broth and agar media (Buncic et al., 1995). The reported results were statistically analyzed.

Greater reduction of microbial numbers is achieved with a combination of both agents than when either is used alone. This interaction between the two treatments is adversely affected by increasing salt concentrations. The use of acid alone is much less effective against *L. monocytogenes* attached onto beef.

The use of bacteriocins for the purpose of extending the shelf life of meat depends on the stability of these proteins in different food systems. Studies carried out using broth systems can be misleading as to the effectiveness of these treatments. For example, in preliminary experiments, a combination of 20,000 units of pediocin AcH and 1.5% lactic acid killed $10^7$ cells of *L. monocytogenes* suspended in peptone water. In meat systems, however, the reduction in cell numbers by the same levels of bacteriocin and acid is at least 2.0 log units lower. Inhibition of *L. monocytogenes* by acid alone was also greater in suspensions than on meat.

Studies in buffered systems indicated a combined effect of nisin with salts of organic acids and curing salts (Buncic et al., 1995). These studies were conducted in buffered broth systems with low concentrations of salt. However, the above-described experiment shows that introduction of low pH and higher salt concentrations reverses this effect as seen. The adverse effect of high salt concentrations on the effectiveness of pediocin and acid in meat is of particular concern in products such as cured meats and some sausages, and there have been no reports of these interactions being studied. Salt is added to several products both to enhance flavor and to inhibit microorganisms. Higher concentrations of bacteriocins may be needed in such products, or the use of other food grade compounds that can stabilize the bacteriocins in such systems. Although these results do not identify the exact nature of interaction between pediocin, salt and acid, they do establish the need for further research into the dynamics of interactions between bacteriocins and food components.
Experiment 3: Characterization of Surface Microflora of Beef that Survive Treatment with Pediocin AcH

Introduction

In the preliminary study it was seen that pediocin AcH application effected an initial reduction in microbial numbers. The surviving bacteria, however, grew quickly to spoilage levels, thereby reducing the extension of shelf life that would have been achieved. This indicates that the surviving bacteria had similar growth characteristics to bacteria that would normally dominate spoilage microflora. This study attempts to identify those species of normal meat microflora that are resistant to pediocin AcH. It is the nature of these resistant organisms that will determine the applicability of pediocin AcH as a preservative agent. While it is expected that most of the resistant species will be gram-negative species (pediocin AcH being a gram-positive bacteriocin), the preliminary tests also indicated that some gram-positive bacteria also survived treatment (as seen by levels of lactic acid bacteria enumerated on MRS agar). This study also addresses the question as to the nature of gram-positive cells that survive pediocin application.

The MIDI-FAME analyses were performed in the research laboratories of Dr. Larry Halverson at Iowa State University under the guidance of his laboratory staff.

Materials and Methods

Treatment of Beef

Ground beef (95% lean) was purchased from different retail stores and stored under refrigeration for no longer than 2 h before treatment. Twenty-five milliliters of pediocin having activity of about 20,000 AU/ml was added to 25 g of each beef sample (giving 10,000 AU/g) and then stomached for 2 min. The resultant slurry was serially diluted with sterile buffered peptone water and spread-plated onto two sets of TSA. One set of plates was incubated at 20°C and the other at 30°C for 4 d. Another 25 g of each beef sample was treated with pediocin (200,000 AU/ml) and the surviving microflora isolated in a similar manner.

Identification of Microflora

Each plate was scored visually for different colony morphologies. Plates incubated at both temperatures were compared and the difference in the distribution of microflora was recorded so as to aid in identification. Isolated colonies were picked for subculture and their relative numbers were recorded. In case of colonies that were present in high numbers (more than 50 colonies per plate), more than one colony of the same morphology were tested. The identification of organisms was carried out using the protocol recommended by Microbial ID, Inc. (MIDI 1996), which involves growth of the organism on TSA agar at 24°C for 22 hours, followed by fatty acid extraction and Gas Chromatography analysis. The MIDI-FAME technique, however, did not always give confirmations on the identity of the organism (particularly the species). In some cases, additional tests had to be
performed to confirm the species of bacterium. These tests included gram staining, test for oxidase, test for catalase, carbohydrate fermentation (glucose, lactose and sucrose), methyl red, Voges-Proskauer, gelatinase, starch hydrolysis, etc (Brock and Madigan, 1991).

Results and Discussion
Five beef samples from three retail stores were tested. The numbers of the microflora ranged from $10^2$ CFU/g to $10^5$ CFU/g. A total of 65 different colonies were isolated from these samples. MIDI FAME analysis provided positive identification of approximately 35% of the organisms at species level. Table 4.7 lists the organisms isolated from beef treated with each level of bacteriocin. Additional tests were required for the identification of the rest. These included gramstains, sugar fermentation tests, tests for catalase, tryptophanase, oxidase and amylase. There are several reviews on the microflora of raw ground meats that provide ample information on the normal microflora present in the samples before the bacteriocin was added (Borch et al., 1996; Gill, 1979).

High frequency occurrence of staphylococci and micrococci were expected in these samples given the extensive handling they had received at the retail stores. The frequency of *Pseudomonas* species was also very high in some of the samples. This may be because the beef was displayed in an aerobic refrigerated environment thus allowing the pseudomonads to grow to high numbers. There was considerable difference in the microflora isolated from beef samples treated with different levels of bacteriocins. While increased levels of bacteriocin activity caused a decrease in the number of staphylococci and micrococci, the *Pseudomonas* and *Acinetobacter* species were not affected as much. Gram-negative organisms are generally not susceptible to pediocin unless their outer membrane is disrupted. Any or all of several factors, including lack of receptors, shielding of cell membrane by LPS layer, and the production of a bacteriocin-degrading enzyme, may play a part in the providing resistance.

Species of *Brevibacterium, Lactobacillus, Lactococcus, Brothrix, Carnobacterium, Chryseobacterium, Flavobacterium* and *Bacillus* were reduced or eliminated by increased levels of bacteriocin. Considering the fact that most of these organisms are natural competitors of *Pediococcus* spp. in meat products, it is not surprising that pediocin was most effective against them. When meat is treated with low levels of pediocin there may not have been enough protein to kill all the susceptible cells. The fact that most of these species occur in lower numbers than the micrococci and staphylococci, may be the reason they were almost completely eliminated with higher levels of bacteriocin. The results of this study indicate that the target spectrum of pediocin AcH is primarily on Gram-positive bacteria many of which dominate meat spoilage microflora. Gram-negative bacteria such as *Pseudomonas* spp. and *Acinetobacter* spp., which constitute a high percentage of the normal spoilage microflora of retail meat, are not affected by the bacteriocin. In meat products treated with pediocin AcH, the elimination or reduction of staphylococci, micrococci and LAB alone may not effect a
Table 4.7. Bacterial species isolated from meat treated with pediocin

<table>
<thead>
<tr>
<th>Relative Number per plate</th>
<th>10,000 AU/g of Pediocin</th>
<th>100,000 AU/g of Pediocin</th>
</tr>
</thead>
<tbody>
<tr>
<td>High numbers</td>
<td>Staphylococcus aureus</td>
<td>Pseudomonas aureofaciens</td>
</tr>
<tr>
<td></td>
<td>Micrococcus luteus</td>
<td>Pseudomonas putida</td>
</tr>
<tr>
<td>20-50 per plate</td>
<td>Pseudomonas aureofaciens</td>
<td>Pseudomonas putida</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas stutzeri</td>
<td></td>
</tr>
<tr>
<td>Lower number</td>
<td>Pseudomonas fluorescens</td>
<td>Pseudomonas fluorescens</td>
</tr>
<tr>
<td>5-20 colonies per plate</td>
<td>Bacillus sterothenophilus</td>
<td>Acinetobacter johnsonii</td>
</tr>
<tr>
<td></td>
<td>Bacillus coagulans</td>
<td></td>
</tr>
<tr>
<td>Very low numbers</td>
<td>Pantoea agglomerans</td>
<td>Pantoea agglomerans</td>
</tr>
<tr>
<td>1-5 colonies per plate</td>
<td>Arthrobacter atrocyaneus.</td>
<td>Arthrobacter atrocyaneus</td>
</tr>
<tr>
<td></td>
<td>Carnobacterium pisciola</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td></td>
<td>Flavobacterium odoratum</td>
<td>Micrococcus luteus</td>
</tr>
<tr>
<td></td>
<td>Chryseobacterium indologenes</td>
<td>Bacillus sterothenophilus</td>
</tr>
<tr>
<td></td>
<td>Brocothrix thermosphacta</td>
<td>Brevibacterium epidermis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brevibacterium iodinum</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bacillus coagulans</td>
</tr>
</tbody>
</table>

Because even susceptible cells can survive treatment if the level of bacteriocin is too low, the initial level of applied bacteriocin has to be carefully estimated so as to kill most or all of the susceptible cells, especially those species that occur in high numbers such as staphylococci and micrococci. Levels of 100,000 AU/g or above of pediocin AcH may be needed for optimum reduction of bacterial numbers in ground beef. This applies particularly to meat in grocery stores that has high populations of bacteria. The level needed would depend on the initial microbial load. It must also be noted that the initial level would also depend on the rate at which bacteriocin is degraded in the meat. It is also possible that bacteriocin-resistant mutants arise.
spontaneously from among the sensitive populations. A method has to be developed to address this problem, such as the inclusion of second bacteriocin.

**Experiment 4: Persistence of Pediocin AcH Activity in Raw and Cooked Meat**

**Introduction**

The application of pediocin to meat surfaces primarily targets the gram-positive bacteria. Though an initial reduction in microbial numbers occurs, the bacteriocin does not control subsequent growth of survivors. This may be due to several factors, one of which is the fact that most of the surviving bacteria are resistant to the bacteriocin, and/or because the levels of bacteriocin were not high enough to target all the sensitive cells. Even when adequate levels of bacteriocin may be added, the protein may be degraded by proteolytic meat enzymes.

The following experiment focused primarily on determining how long bacteriocin activity persists in meat. Since bacterial enzymes can also cause the degradation of the bacteriocin, the difference in initial microflora could introduce a high degree of variation to the experiment. Therefore, the first part of the experiment was conducted on raw beef that had been irradiated to eliminate bacterial cells. Thus, only naturally occurring meat enzymes were presumed to cause the degradation of the bacteriocin after the initial fall in activity due to non-specific binding to meat proteins has occurred. In a further attempt to understand the dynamics of the degradation, the study was conducted over three different temperatures. Meat treated with pediocin AcH was stored at freezing, refrigeration and ambient temperatures.

One of the most common methods of food preservation is heat treatment. Since pediocin AcH is known to be resistant to temperatures up to 100°C, it can be incorporated into products before they are cooked. With respect to this study on pediocin application, heat treatment has a dual significance. Firstly, it inactivates enzymes that can degrade the bacteriocin. Secondly, it kills most of the microflora in the meat that would otherwise grow and contribute to the further loss of pediocin activity (through binding and degradation). Also, heat treatment shifts the spoilage microflora towards the more heat tolerant/resistant gram-positive bacteria. Gram-negative bacteria, the sub-population more likely to survive bacteriocin treatment, are much more susceptible to heat. This part of the study was conducted over two storage temperatures. Frozen storage was not studied, as the initial results with raw meat indicated a very long shelf life under frozen conditions.

Earlier experiments have raised concerns about targeting the bacteriocin-resistant species of bacteria, most of which are gram negative. The addition of acid was one of the methods explored in these studies. In this experiment heat treatment was explored as a way to shift the microbial population towards susceptible gram positive bacteria. In keeping with that same objective, irradiation and high pressure were also studied as possible technologies to be used in conjunction with bacteriocin application. While studies have shown that a combination of high hydrostatic pressure and bacteriocin is effective against gram-negative cells these studies do not mention if the pressure affects the bacteriocin itself, i.e., if there is a drop in bacteriocin activity due to the
treatment, nor is there any information available on the effect of irradiation on bacteriocins. This is especially significant when initial levels of bacteriocin to be applied are estimated.

Materials and Methods

Loss of Pediocin Activity in Meat:

Ground beef was obtained from various retail stores and 2.5 g samples placed in sterile bags and frozen. Then 2.5 ml of semi-purified pediocin solution was added to the meat, the bags sealed and irradiated at 7 kGy. This level of irradiation was chosen as it is the maximum allowed for frozen meat. The samples were then divided into three lots. One set of samples was stored at 25°C (room temperature), one set at 7°C (refrigeration) and one at -25°C (frozen). Some of the frozen samples were removed from -25°C after 24 days and stored at 7°C. These samples were also removed at intervals during storage, stomached for 1 min, centrifuged, and the supernatant assayed for pediocin (as described in the preliminary experiment).

In the second part of the experiment the degradation of pediocin AcH in cooked meat was studied. Beef samples were prepared as outlined above. After being sealed the bags were placed in a water bath at 75°C for 5 minutes. The samples were immediately cooled in cold water (3-5°C) and divided into two sets. One set was stored at refrigeration temperature (4°C) and the other at room temperature (25°C). Samples were removed at intervals during storage, stomached for 1 min, centrifuged and the supernatant assayed for pediocin activity.

Effect of High Pressure and Irradiation

5 ml samples of semi-purified pediocin were placed in sterile bags and irradiated at doses of 0.5, 2.0, 3.5, 5.0 and 7.0 kGy. The samples were then assayed for pediocin activity. Five milliliter samples of semi-purified pediocin were double bagged and sealed in sterile irradiation bags. They were then subjected to pressures of 30 kpsi and 100 kpsi (at room temperature). The samples were then assayed for pediocin activity as described in the preliminary experiment.

Results and Discussion

Degradation of Bacteriocin in Meat

In every case 75% of the bacteriocin activity was lost within minutes of addition to the meat irrespective of the initial amount. This may be attributed to the binding of the bacteriocin to various meat proteins. Because the meat was irradiated at 7 kGy, and no detectable bacterial growth occurred during storage, the reduction in recoverable bacteriocin activity was ascribed mainly to proteolysis caused by meat enzymes. The recoverable bacteriocin activity fell to levels below 1% of that added to the meat after 4 days when stored at 25°C, and after 15 days at 7°C (Fig. 4.13). In frozen samples the bacteriocin levels remained high even after 3 months. This again could be related to enzyme activity. Most of the proteolytic enzymes in meat have higher activity at higher temperatures (30-37°C). The freezing of meat further restricts the movement of enzyme and bacteriocin thereby minimizing their interaction and rate of activity. Since different stores used different grind
sizes, considerable variations were seen in samples in terms of average particle size. The rate of loss of pediocin activity was lower in samples with larger particle size. This is probably because the increased surface area of samples with smaller particles allows greater area of contact between the bacteriocins and meat proteases. The greater surface area of finer particles also provides for greater release of proteolytic enzymes into the samples.

For frozen samples that were moved to refrigeration temperature after 24 days, the bacteriocin degradation followed a similar curve to that of samples refrigerated throughout. This experiment was designed to mimic temperature abuse during frozen storage, especially on the surfaces of large cuts of meat. The bacteriocin, however, persisted longer in the samples that were not previously frozen. Bacteriocin activity in raw beef decreased from approximately 18.75% to 0.5% over a period of 17 days in case of frozen and thawed samples and 21 days in case of refrigerated samples (Fig. 4.13). The increase in the rate of degradation of pediocin in frozen and thawed samples may be due to the release of intracellular enzymes due to cell membrane damage during freezing. It is also possible that increased water activity in frozen-thawed samples also contributes to increased movement and interaction of the enzymes and pediocin in the samples.

In cooked samples the bacteriocin activity persisted for more than 98 days at 4°C and an average of 6 days at room temperature (Fig. 4.14). The degradation was ascribed more to bacterial activity in this case, as several Gram-positive organisms are capable of surviving the heat treatment at 75°C, which would denature most of the proteolytic enzymes in the meat itself. It is also possible that the denaturation of meat proteins by heat prevents them, to some extent, from binding to pediocin. Gram-negative bacteria are more susceptible to heat treatment, but any surviving cells would also grow in the meat and cause degradation of the pediocin.

Effect of Irradiation and High Pressure Treatment

The bacteriocin activity remained unaffected by all levels of irradiation (up to 7.0 kGy) and high hydrostatic pressure (up to 100 kpsi). Samples with different levels of activity were tested. In each case the activity of treated samples remained the same as the controls as indicated in Table 4.7. However, the fact that the assay method tests a two-fold dilution series, small changes in activity cannot be detected.

<table>
<thead>
<tr>
<th>Table 4.7. Effect of irradiation and high pressure on bacteriocin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteriocin activity of treated sample</strong></td>
</tr>
<tr>
<td><strong>Sample Number</strong></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
</tbody>
</table>
Av-F: Frozen samples. Av-R: Samples stored at 4°C. Av-A: Samples Stored at 25°C
Av-r: Frozen samples that were later transferred to 4°C (arrow shows time of transfer)

Fig. 4.13. Degradation of Pediocin AcH over time in raw ground beef

RmT: Samples stored at 25°C, Ref: Samples Stored at 25°C

Fig. 4.14. Degradation of Pediocin AcH over time in cooked ground beef
It was noticed that samples that showed the earliest decrease of bacteriocin activity had a strong putrefactive odor as compared to other samples that had a more 'sour' odor. This may be because the proliferation of bacteria that cause protein and amino acid degradation caused a more rapid degradation of the bacteriocin. However, this cannot be confirmed because the identification of the spoilage bacteria in each sample and correlation to rate of loss of bacteriocin activity was not carried out.

In a typical meat system, the bacteriocin activity is affected by several factors. The most important one is enzymatic degradation by both meat tissue and bacterial enzymes (Bhunia et al., 1988; Ray and Daeschel, 1992). Another important factor affecting bacteriocin activity is the binding of the bacteriocin with other proteins. Both these factors must be taken into account when determining how much bacteriocin is to be added to any product (Holzapfel, 1995). The above results confirm that pediocin AcH persists in raw and cooked meat long enough to effect control of microbial growth. Also the bacteriocin is compatible for use with irradiation and high pressure. This can be used to further develop a method of extending the shelf life of meat that targets both bacteriocin susceptible and resistant bacteria. The duration of time for which bacteriocin activity persists in raw and cooked meats stored at various temperatures is an important consideration in the development of such methods.

**Experiment 5: Effect of EDTA, Maltol, Triton-X 100 and Tween-80 on the Inhibitory Activity of Pediocin AcH against Salmonella typhimurium DT104 and Escherichia coli 0157:H7**

**Introduction**

In preliminary experiments and in Experiment 3, results indicated that pediocin AcH had little or no effect on gram-negative bacteria. This poses a major drawback to the use of bacteriocin as a preservative agent for meat as gram-negative organisms such as Pseudomonas spp. and Acinetobacter spp. constitute a major part of spoilage microflora of meat. Thus any method developed for the extension of meat shelf life has to include gram-negative spoilage species in its list of targeted organisms. From the perspective of this study, this can be achieved in three ways. The gram-negative species can be targeted with other preservative methods or agents that can be used in conjunction with bacteriocin (that do not affect its activity on the gram-positive target organisms). This may be achieved by the use of organic acids, heat treatment, irradiation and high hydrostatic pressure. The use of modified atmosphere packaging (vacuum and CO\textsubscript{2}) can inhibit the growth of gram-negatives while encouraging the growth of gram-positive bacteria. Finally, other chemical agents may be used that render gram-negative bacteria susceptible to bacteriocins.

Previous studies have demonstrated that sub-lethal injury rendered several gram-negative bacteria such as Aeromonas hydrophila, Salmonella typhimurium, Yersinia enterocolitica, Escherichia coli, Pseudomonas fluorescens and P. putida, and some Gram-positive bacteria that are otherwise resistant to bacteriocins of LAB,
sensitive to pediocin (Kalchayanand et al., 1992). In case of gram-negative cells, the LPS layer protects the cell by preventing access of the pediocin into the cell membrane. Sublethal injury or ion chelators disrupt the LPS layer thereby allowing the bacteriocin access to the cell membrane (Schved et al., 1994a, b). The most commonly used ion-chelator in these studies is ethylene diamine tetraacetate (EDTA). Some studies have also reported that maltol (4-hydroxy-pyrone) has the same effect.

A combination of ion-chelator, bacteriocin and a detergent has been recommended to achieve the broadest range of target bacteria. Patents have been issued for the use of a combination of Nisin, EDTA and Triton X 100 as a sanitizer for various food contact surfaces (Blackburn et al., 1989). It is theorized in the patent applications that ion-chelators disrupt the cell surfaces so as to better deliver the bacteriocin to the cell membrane while the detergent helps stabilize the bacteriocin in solution and better target the amphiphilic cell membranes. Based on this idea, the experiment was designed to test if a similar enhancement of anti-microbial activity could be achieved against gram-negative bacteria. This experiment focused primarily on finding the combination of agents that effected the greatest reduction in bacterial numbers.

One of the issues that needs to be addressed when developing an anti-microbial agent for food preservation is its effectiveness in killing and controlling the growth of pathogens. While earlier experiments have demonstrated the effect of pediocin AcH against *L. monocytogenes* (a major meat-borne pathogen), its effect against gram-negative pathogens (on meat) had not been studied. *Escherichia coli* 0157:H7 and *Salmonella typhimurium* DT104 were used in this study, because they are both major gram-negative pathogens associated with meat and meat products. Both organisms are present in low levels in meat and meat product and have low infective doses. Therefore, even a small reduction in numbers would significantly reduce the risk of foodborne illness.

**Materials and Methods**

**Inoculation and Treatment of Meat**

Lean beefsteak was cut into cubes (1 cm x 2 cm x 1 cm) and irradiated at 10 kGy. This was carried out at the Iowa State Linear Accelerator Facility. The beef cubes were stored on ice until use. They were then washed in sterile distilled water (at room temperature) and inoculated by a 20 min immersion in a culture of *S. typhimurium* DT104 or *E. coli* 0157:H7, diluted to $10^8$ CFU/ml with 0.1% peptone water (at 37°C). After inoculation, the cubes were immersed in 100 ml of their respective treatment solutions and shaken gently for 10 min. Tow to three cubes (based on their weight) were used for each treatment in each experiment. Each experiment was performed twice.

**Formulation of Treatment**

Treatments used are listed in Table 4.8a and various combinations of these treatments used are listed in Table 4.8b. The bacteriocin was prepared as described in Experiment 1.
Table 4.8. Description of treatments and treatment combinations applied

a. Treatments applied

<table>
<thead>
<tr>
<th>Test Name</th>
<th>Treatment Initial</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>C</td>
<td>Immersion in sterile 50mM sodium chloride solution</td>
</tr>
<tr>
<td>Peidiocin</td>
<td>P</td>
<td>100,000 AU of pediocin AcH *</td>
</tr>
<tr>
<td>Maltol</td>
<td>M</td>
<td>40mMof Maltol*</td>
</tr>
<tr>
<td>EDTA</td>
<td>E</td>
<td>20mM solution*</td>
</tr>
<tr>
<td>Triton X 100</td>
<td>X</td>
<td>1% solution*</td>
</tr>
<tr>
<td>Tween 80</td>
<td>T</td>
<td>1% solution*</td>
</tr>
</tbody>
</table>

b. Treatment combinations applied

<table>
<thead>
<tr>
<th>Treatment Combinations applied</th>
<th>Initials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pediocin, EDTA and Tween</td>
<td>PET</td>
</tr>
<tr>
<td>Pediocin Maltol and Tween</td>
<td>PMT</td>
</tr>
<tr>
<td>Pediocin, EDTA and Triton X 100</td>
<td>PEX</td>
</tr>
<tr>
<td>Pediocin Maltol and Triton X 100</td>
<td>PMX</td>
</tr>
<tr>
<td>Pediocin, EDTA, Maltol and Triton X 100</td>
<td>PEMX</td>
</tr>
<tr>
<td>Pediocin, EDTA, Maltol and Tween 80</td>
<td>PEMT</td>
</tr>
</tbody>
</table>

* All chemical agents were dissolved in 50 ml of 50mM sodium chloride in the desired quantities, the pediocin added as needed and the remaining volume made up to 100 ml with the sodium chloride solution.

Enumeration of Survivors

After treatment, the cubes were placed in stomacher bags and diluted two-fold with 1% peptone water. The samples were stomached for 2 min and the resulting homogenate serially diluted as needed and plated on TSAYE. The plates were incubated at 37°C for 2 d.

Results and Discussion

The results of the test are plotted in Fig. 4.15. Pediocin alone effected a small reduction in bacterial numbers (approximately 0.2 log units). This could be because a small percentage of the population of cells was injured and therefore susceptible to the bacteriocin. The combination of pediocin, EDTA and Triton X-100 effected a reduction in bacterial numbers of only 0.5 Log units. The use of Maltol in the treatments did not have as great an anti-bacterial activity as EDTA, which proved to be superior in this respect. The greatest reduction effected was roughly 0.8 log units in case of E. coli 0157: H7 and about 0.5 log units in case of S. typhimurium DT104.
Fig. 4.15. Effectiveness of various treatment combinations against *E. Coli* and *Salmonella*
The use of EDTA and Maltol in combination with bacteriocin has been reported to cause much higher reductions in cell numbers in broth studies (Kang and Fung, 1997). However, when tested on meat systems these effects have not been observed. As demonstrated by these results, the anti-microbial activity of ion chelators and bacteriocins against gram-negative bacteria is very limited in meat systems. When compared to initial reductions by various treatments in previous experiments, this appears inadequate. However, considering the fact that both pathogens occur at very low numbers in meat and have very low infective levels, every hurdle to microbial growth, however, minor, becomes significant. In terms of shelf-life extension, however, these treatments in themselves did not prove sufficiently effective against the organisms tested. However, keeping in mind that these tests were carried out with pure cultures, the treatments may prove to more effective against meat surface microflora. Also the tests used *E. coli* 0157:H7 and *S. typhimurium* DT104, both of which are not typical meat spoilage organisms and rarely grow to high numbers in competition with spoilage microflora.

While the use of ion-chelators and detergent may have a synergistic action with pediocin by way of helping deliver the bacteriocin to its target site, it is possible that the use of a physical agent (such as high pressure) may further enhance this anti-bacterial action. There is an added advantage to using agents such as EDTA and Maltol in that they are already approved for use in foods. Maltol has a very strong odor and may not be able to be used in many products. EDTA, however, may find application in a broader range of products.

**Experiment 6: Shelf-Life Extension of Raw and Cooked Meat using Various Combinations of Pediocin AcH, Nisin, EDTA, Triton X-100, Acetic Acid, Irradiation and High Hydrostatic Pressure**

**Introduction**

In previous experiments, it was established that pediocin AcH had potential to be used in the shelf life extension of meat. When added in adequate levels, it effected a significantly high reduction in initial numbers of gram-positive bacteria. The bacteriocin persisted in meat (both cooked and raw meat) for a duration of time that allowed for its use as an agent against spoilage caused by temperature abuse. The major drawback in the application of this protein was its ineffectiveness against gram-negative bacteria. This can, however, be addressed by the use of other preservative agents to target that sub-population. Pediocin may be used in combination with organic acids, irradiation, high pressure and chemical agents such as EDTA and triton-X-100.

Pediocin AcH was seen to be highly effective in killing cells of *Listeria monocytogenes* attached to meat (Experiment 2). It was also effective (to a small extent) against gram-negative pathogens such as *E. coli* 0157: H7 and *S. typhimurium* DT104 when used in combination with EDTA and Triton X-100 (Experiment 5). This effect against pathogenic bacteria increases its potential for use as a preservative agent in foods. In this experiment, the bacteriocin-resistant bacteria have been targeted by a combination of physical, chemical and biological agents. Pediocin has been combined with acetic acid, EDTA and Triton X-100, high hydrostatic
pressure and irradiation. Both high pressure and irradiation affect total microflora. However, gram-negative bacteria are much more susceptible to these treatments than gram-positive bacteria (Thayer, 1995). Modified atmosphere packaging has been introduced to further shift the microbial population to bacteriocin sensitive gram-positive lactic acid bacteria (Nissen et al., 1996). Also, in order to address the possibility of resistant mutants arising from pediocin susceptible populations, another bacteriocin (nisin) was added. Previous experiments have indicated that this reduces the frequency of occurrence of resistant mutants (Hanlin et. al., 1993) This is due to the fact that the main mechanisms of resistance to bacteriocin are altered composition of cell membrane and the production of bacteriocin degrading enzymes (e.g., nisinase). Since both these mechanisms affect specific types of molecules, the use of two different types of bacteriocins (e.g., a lantibiotic and a non lantibiotic) reduces the probability that the cell can develop resistance to either one or both.

Fig. 4.16 shows the various ways in which the dynamics of growth of spoilage microflora can be manipulated so as to achieve an extension of shelf life. Different treatments affect growth parameters in different ways. The effect of pediocin, as previously seen, was to decrease the initial number of cells (B) and any residual pediocin activity would also affect an extension of the lag phase. The effect of adding acid to meat was primarily initial reduction in numbers and extension of the lag period, as the cells take longer to adapt to growth at low pH (C). The various treatment combinations tested in this experiment attempt at further affecting spoilage rate by altering the rate of growth as well. A shift of microflora to a slower growing one that attains stationary phase at lower numbers would achieve the greatest extension of shelf life.

Statistical modeling of the data (using a non-linear regression models) was performed with the specific aim of analyzing the nature of change effected by each treatment. No previous study reported in the literature has used this statistical approach to study spoilage populations in food systems.

Materials and Methods

Formulation of Treatments

The seven treatment combinations applied to the meat cubes in this experiment are listed in Table 4.9.

<table>
<thead>
<tr>
<th>Treatment combinations</th>
<th>Initials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, (100 mM sodium chloride)</td>
<td>C</td>
</tr>
<tr>
<td>Bacteriocin and acetic acid</td>
<td>PA</td>
</tr>
<tr>
<td>Bacteriocin, acetic acid, Triton X-100 and EDTA</td>
<td>PETA</td>
</tr>
<tr>
<td>Bacteriocin and acetic acid, followed by irradiation</td>
<td>PAI</td>
</tr>
<tr>
<td>Bacteriocin, acetic acid, Triton X-100 and EDTA followed by irradiation</td>
<td>PETAI</td>
</tr>
<tr>
<td>Bacteriocin and acetic acid followed by pressurization</td>
<td>PAH</td>
</tr>
<tr>
<td>Bacteriocin, acetic acid, Triton X-100 and EDTA, followed by pressurization</td>
<td>PETAH</td>
</tr>
</tbody>
</table>
Fig. 4.16. Strategies For Shelf Life Extension

A - Normal pattern of microbial growth,  B - Growth from decreased initial numbers  C - Growth from lower initial numbers and longer lag phase  D - Growth with lower initial numbers, longer lag phase and lower slope  E - Growth with the same parameters as D, but with a lower number at stationary phase.
Each of the treatments with bacteriocins contained 800,000 AU/ml of pediocin AcH and 200,000 AU/ml of Nisin. Acetic acid was applied at 1%, EDTA at 20 mM and Triton X-100 at 1%. Irradiation (e-beam) was at 3 kGy average absorbed dose and high-pressure at 75,000 psi for 5min. All treatments listed in Table 4.9 were dissolved in 100mM sodium chloride. Pediocin was prepared as outlined in earlier experiments. One single batch was prepared and used for all repetitions so as to ensure identical bacteriocin concentration and activity. The experiment was repeated twice.

**Treatment of Meat**

Lean beefsteaks were obtained from various retail stores and aseptically cut into cubes (1 cm x 2 cm x 1 cm in size). The cubes were washed in sterile distilled water, divided into seven sets of twenty each and immersed completely in the treatments for 10 min at room temperature. The cubes were then removed aseptically and individually placed in petridishes. Those that required high-pressure treatments were first transferred from the treatment liquid into sterile bags, double-bagged, sealed and pressurized. The cubes were then removed from the bags and placed in petridishes. The petridishes were then vacuum packaged individually and stored at 15°C.

In the study using cooked meat, the samples were prepared and treated as outlined above. The meat cubes were removed after immersion in the various treatments, placed in bags, sealed and immersed in a water bath at 80°C for 3 min. This temperature was chosen as it mimics the temperature used in the production of some processed meats with a long shelf life. Also the higher temperature and lower time is more effective at degrading meat proteases that would otherwise affect bacteriocin activity. The cubes were then removed, placed in petridishes, vacuum-packaged and stored. Samples requiring irradiation were refrigerated after being placed in petridishes, taken to the irradiation facility immediately and irradiated prior to vacuum packaging.

**Enumeration of Bacteria**

Each meat cube was weighed in a stomacher bag, peptone water added to make up a 1/10 dilution and stomached for 2 min. The resulting slurry was then serially diluted and spread plated in duplicate using a spiral plater. The samples were plated onto tryptic soy agar (TSA), MRS agar and violet red bile glucose agar (VRBG) to enumerate total aerobic, lactic acid spoilage and gram-negative populations respectively. The TSA plates were incubated at 20°C for 4 d, the MRS were incubated anaerobically at 30°C for 4 d and the VRBG plates at 37°C for 2 d.

**Statistical Analysis**

The increase in bacterial numbers over time was analyzed using SAS. A non-linear growth model using Gompertz (1825) equation was fit onto each set of data. This equation is commonly used to describe growth of biological populations.
The Gompertz equation is:

\[ N = N_0 e^{\left(\beta_0 \left(1 - e^{-\alpha t}\right) / \alpha\right)} \]

where \( N \) is the log CFU/g in the sample, \( N_0 \) is the initial number of cells and \( t \) is the time (in days) of storage, \( \alpha \) is the decay in specific growth rate of the population and \( \beta \) is the constant of proportionality (Ocku, 1996). The basic assumptions of this model are:

1. The substrate is non-limiting and the process is always saturated with substrate.
2. The growth is proportional to dry weight \( W \), with constant of proportionality \( \beta \).
3. The effectiveness of growth decays exponentially with time according to first order kinetics.

According to assumption 3,

\[ \frac{d\beta}{dt} = -\alpha t \]

Then, \( \beta = \beta_0 e^{-\alpha t} \)

Substituting this in the equation \( \frac{dW}{dt} = \beta W \) (Assumption 2),

\[ \frac{dW}{dt} = \beta_0 W e^{-\alpha t} \]

Integrating, this equation gives:

\[ W = W_0 e^{\left(\beta_0 \left(1 - e^{-\alpha t}\right) / \alpha\right)} \]

Assuming dry weight is directly proportional to the number of cells (2), \( W \) can also be substituted with \( N \) where \( N = W/\text{weight of each cell} \).

The inflection point of the curve is given by the co-ordinates \((t^*, N^*)\) where \( t^* = 1/\alpha \text{Ln} (\beta_0/\alpha) \) and \( N^* = N_f/\alpha \) (\( N_f \) is the final number at stationary phase). As seen in Fig. 4.17 the population goes through a phase of increasing growth rate until it reaches a phase of constant growth (exponential/log phase) after which it undergoes decreasing growth until it reaches stationary phase. The point of inflection is where the increasing and decreasing exponential curves meet (i.e., where \( dN/dt = 0 \)).

The values of \( N_0, \beta_0 \) and \( \alpha \) determine the characteristic of the curve in the following ways:

1. The greater the value of \( N_0 \) the greater the initial number of cells. Thus, a comparison of \( N_0 \) values of various treatments to that of the control gives the initial reduction of microbial numbers.
2. The greater the value of \( \beta_0 \), the greater the value of \( N_f \), i.e., the number of cells at stationary phase.
   Conversely smaller the value of \( \beta_0 \), the lower the maximum population density.
3. When comparing two curves, the one with the greater value of \( \alpha \) (when all other parameters remain the same) will have a higher slope at exponential growth phase, i.e., that curve will have a shorter period of time between when it reaches log phase and exits it.
4. If \( \alpha < 0 \) and \( \beta_0 > 0 \), then \( N_f \rightarrow \infty \) at \( t \rightarrow \infty \), i.e., the curve continues increasing indefinitely with time.
Fig 4.17. Curve parameters as described by Gompertz Equation
5. If \( \alpha < 0 \) and \( \beta_o < 0 \), the \( N_f \to \infty \) at \( t \to \infty \), i.e., the curve begins to decrease with time and the population dies in time.

6. If \( \alpha < 0 \) and \( \beta_o < 0 \), the \( N_f \to N_0 e^{\beta_o / \alpha} \) at \( t \to \infty \), i.e., the final number of the population is lower than the initial one indicating a decreasing trend.

The curves of the different treatments were then compared for differences in estimated values of \( N_0, \beta_o \) and \( \alpha \). Treatments with lower values of \( N_0 \) had lower initial numbers of bacteria, i.e., a greater antimicrobial activity, those with lower \( \beta_o \) values reached stationary phase at lower numbers indicating a shift in the composition of microflora. When treatments with similar initial numbers and \( \beta_o \) values differed with respect to the \( \alpha \) value, it indicated that the treatment with the higher value of \( \alpha \) had a higher slope and, therefore, a faster growth rate. This change in growth rate could be caused by both the shift of microflora to slower growing organisms, the residual inhibitory effect of the treatment applied or a combination of both. Also, in order to estimate how well the non-linear model fit the observed data, the studentized residuals were plotted for each curve. The plots were then examined for any evidence of a pattern that deviates from \( N \sim (0, 1) \), i.e., a normal distribution. In addition, the average values of populations were also plotted so as to get a rough idea of growth trends and how they were affected by various treatments.

The above-described analysis was not conducted on cooked meat samples. As the experiments progressed, it was noted that the numbers of bacterial populations in most cooked samples did not increase sufficiently to be effectively analyzed in this fashion.

**Results and Discussion**

**Effect of Treatments on Raw Meat**

Total aerobic plate counts of control samples of raw meat reached levels of \( 10^6 \) CFU/g within 4 d on average (Fig. 4.18). Aerobic plate counts of samples treated with bacteriocins and acid alone (PA) reached the same level in approximately 9 d and those given treatment PETA in 15 to 18 d. In all other samples, the counts did not reach \( 10^6 \) CFU/g over the period of study (18 days). While treatments PA and PETA effected a considerable extension of shelf life of the meat (4 and 12 days), treatments involving irradiation and high pressure were much more effective (bacterial numbers did not reach spoilage levels). Also treatments using EDTA and Triton X-100 had lower microbial numbers than treatments without those chemicals. The initial reduction in microbial numbers was not greatly affected by the addition of these agents (not more that about 0.5 log), but the other attributes of the growth curve were altered.

The combination of chemical and biological agents with high pressure was much more effective in controlling microbial growth than treatments using irradiation (Figs. 4.18 and 4.19). This may be due to a synergistic action between bacteriocins and high pressure where the pressure helps the bacteriocins penetrate into the cell membranes of the microorganisms. Fig. 4.18 indicates the average values of total aerobic bacterial numbers as plotted against days of storage. The difference between the bacterial counts of meat
Fig. 4.18. Effect of various treatment combinations on total aerobic microflora of raw beef

Fig. 4.19. Effect of various treatment combinations on the lactic acid bacteria on raw beef
sample treated with PA and PETA illustrates the enhanced effect of bacteriocins when used with EDTA and Triton X-100.

Similar trends were observed in case of lactic acid bacterial (LAB) populations enumerated on MRS agar (Fig. 4.19). However, the difference between treatments with EDTA and Triton X-100 (PETA, PETAI, PETAH) and those without (PA, PAI, PAH) was not as great as in case of total aerobic plate counts. This may be because the addition of EDTA and Triton X-100 enhances the effect of bacteriocins on Gram-negative bacteria more than on Gram-positives. The fact that the lactic acid bacterial populations closely follow the total plate counts indicates that a majority of the organisms growing in the meat were LAB. Fig. 4.19 illustrates the effect of various treatments on the growth of lactic acid bacteria in meat.

In case of Enterobacteriaceae populations enumerated on VRBG agar, the bacterial numbers were much lower than in case of LAB or total aerobic microflora. However, the same trends in treatment effects were observed. These effects are illustrated in Figure 4.20. There was a greater difference between treatments PAH and PETAH than in case of TSA and MRS counts which again points to a greater effect of EDTA and Triton X-100 on Gram-negative bacteria than Gram-positives.

The synergistic action between pediocin AcH, Nisin and high-pressure may be due to their their concerted action on the cell membrane. EDTA chelates divalent ions that are needed to stabilize the LPS layer of Gram-negative bacteria. Hence, the bacteriocins have better access to the cell membrane. The application of pressure adds to the stress on the cells and also aids in the delivery of bacteriocin to its target site. Both pediocin AcH and Nisin are hydrophobic proteins and the application of pressure may increase their affinity for hydrophobic areas of the cells such as phospholipid bilayers of the cell membranes (Kalchayanand et al., 1994, 1998).

Triton X-100 may act in several ways. It may help stabilize the pediocin in solution, thereby maintaining it in active conformation. It may also help deliver the pediocin to the cell membrane, or minimize the binding of pediocin to other proteins, or the formation of aggregates.

In case of treatment of the meat with irradiation, the resistant cells surviving treatment with bacteriocins and other chemicals are then subjected to further stress. The antimicrobial effect of ionizing radiation is primarily due to DNA damage by both electrons and free radicals. While Gram-negative cells are more susceptible to radiation damage, those that are sublethally injured may recover and grow. In short, radiation targets the survivors of the other treatments but does not enhance the effect of the same.

**Statistical Analysis**

Table 4.10 lists the estimates calculated for $N_0$ and $\alpha$ and $\beta$. The standard error of each estimate is also given in parentheses. The estimates differed significantly both among different populations and among curves fitted for different treatments.

Analysis of the trends revealed by the fitted curves indicates that treatment with PA and PETA causes an initial reduction in both cell numbers, and in final populations of the total aerobic microflora of the meat.
Fig. 4.20. Effect of various treatment combinations on enterobacteriaceae in raw beef
Table 4.10: Estimates of $N_0$, $\alpha$ and $\beta_0$ for bacterial populations from various treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$N_0$ estimate</th>
<th>$\alpha$ estimate</th>
<th>$\beta_0$ estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>5.65 (0.60)</td>
<td>0.01 (0.28)</td>
<td>0.10 (0.09)</td>
</tr>
<tr>
<td>PA</td>
<td>3.21 (0.48)</td>
<td>0.04 (0.06)</td>
<td>0.11 (0.05)</td>
</tr>
<tr>
<td>Total</td>
<td>3.41 (0.52)</td>
<td>-0.03 (0.07)</td>
<td>0.04 (0.03)</td>
</tr>
<tr>
<td>aerobic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>1.75 (0.30)</td>
<td>0.05 (0.04)</td>
<td>0.12 (0.04)</td>
</tr>
<tr>
<td>Microflora</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PETA</td>
<td>1.13 (0.20)</td>
<td>0.07 (0.03)</td>
<td>0.16 (0.05)</td>
</tr>
<tr>
<td>PAH</td>
<td>2.22 (0.35)</td>
<td>-0.27 (0.17)</td>
<td>0.002 (0.005)</td>
</tr>
<tr>
<td>PETAH</td>
<td>1.91 (0.24)</td>
<td>-0.51 (0.48)</td>
<td>0.000 (0.0003)</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>0.74 (0.27)</td>
<td>-0.02 (0.05)</td>
<td>0.10 (0.06)</td>
</tr>
<tr>
<td>PETA</td>
<td>0.71 (0.30)</td>
<td>0.03 (0.06)</td>
<td>0.12 (0.09)</td>
</tr>
<tr>
<td>PAH</td>
<td>0.91 (0.40)</td>
<td>0.05 (0.09)</td>
<td>0.12 (0.11)</td>
</tr>
<tr>
<td>PETAH</td>
<td>1.02 (0.22)</td>
<td>-0.15 (0.12)</td>
<td>0.01 (0.02)</td>
</tr>
<tr>
<td>Lactic acid bacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PETA</td>
<td>2.89 (0.57)</td>
<td>0.004 (0.08)</td>
<td>0.06 (0.04)</td>
</tr>
<tr>
<td>PAI</td>
<td>1.31 (0.37)</td>
<td>-0.01 (0.06)</td>
<td>0.08 (0.05)</td>
</tr>
<tr>
<td>MRS -PAH</td>
<td>1.12 (0.44)</td>
<td>0.04 (0.09)</td>
<td>0.11 (0.09)</td>
</tr>
<tr>
<td>MRS -</td>
<td>0.84 (0.30)</td>
<td>-0.02 (0.08)</td>
<td>0.07 (0.06)</td>
</tr>
</tbody>
</table>

(Figs. 4.21, and 4.22). In case of lactic acid bacteria (LAB) the same trend was seen although the reduction in final populations (indicated by $\beta_0$) is not as much as in the case of total microflora (Figs. 4.25 and 4.26). This may indicate that the population of lactic acid bacteria is not affected qualitatively by PA and PETA treatments as much as it is affected quantitatively (immediately after application). The treatments cause a greater decrease in the initial numbers of LAB but do not change the composition of the microflora. Therefore, the surviving LAB grow to the same levels as the control.

In case of gram-negative bacteria, it is evident that the application of PETA results in a large reduction of initial and final numbers, and that the growth rate is also significantly affected (Figs. 4.29 and 4.30). This
Fig. 4.21. The fitted regression curve for treatment (TSA-CON) and the corresponding residual plot

The parameter estimates were: $\alpha = 0.0107$, $\beta = 0.1038$ and $N_0 = 5.6509$.
The residual plot indicates no pattern to distribution of residuals.

Fig. 4.22. The fitted regression curve for treatment (TSA-PETA) and corresponding residual plot

The parameter estimates were: $\alpha = -0.0309$, $\beta = 0.0355$ and $N_0 = 3.410$.
The residual plot indicates no pattern to distribution of residuals.
Fig. 4.23. The fitted regression curve for treatment (TSA-PETA) and the corresponding residual plot

The parameter estimates were: $\alpha = 0.0482$, $\beta = 0.1256$ and $N_0 = 1.2140$.

The residual plot indicates no distinct pattern to distribution of residuals.

Fig. 4.24. The fitted regression curve for treatment (TSA-PETAH) and the corresponding residual plot

The parameter estimates were: $\alpha = -0.2368$, $\beta = 0.003$ and $N_0 = 1.8198$.

The residual plot indicates no distinct pattern to distribution of residuals.
Fig. 4.25. The fitted regression curve for treatment (MRS-CON) and the corresponding residual plot
The parameter estimates were: $\alpha = 0.3933$, $\beta = 0.3352$ and $N_0 = 3.685$. The residual plot indicates no distinct pattern to distribution of residuals.

Fig. 4.26. The fitted regression curve for treatment (MRS-PETA) and the corresponding residual plot
The parameter estimates were: $\alpha = 0.1227$, $\beta = 0.2430$ and $N_0 = 1.1342$. The residual plot indicates no distinct pattern to distribution of residuals.
Fig. 4.27. The fitted regression curve for treatment (MRS-PAI) and the corresponding residual plot

The parameter estimates were: \( \alpha = -0.0223, \beta = 0.0964 \) and \( N_0 = 0.7377 \).
The residual plot indicates no distinct pattern to distribution of residuals.

Fig. 4.28. The fitted regression curve for treatment (MRS-PETAH) and the corresponding residual plot

The parameter estimates were: \( \alpha = -0.154, \beta = 0.0104 \) and \( N_0 = 1.021 \).
The residual plot indicates a definite pattern to distribution of residuals.
Fig. 4.29. The fitted regression curve for treatment (VRBG-CON) and corresponding residual plot

The parameter estimates were: $\alpha = 0.0587$, $\beta = 0.1049$ and $N_0 = 5.2188$.
The residual plot indicates no pattern to distribution of residuals.

Fig. 4.30. The fitted regression curve for treatment (VRBG-PETA) and the corresponding residual plot

The parameter estimates were: $\alpha = 0.0039$, $\beta = 0.0549$ and $N_0 = 2.8863$.
The residual plot indicates a pattern to distribution of residuals.
Fig. 4.31. The fitted regression curve for treatment (VRBG-PAI) and the corresponding residual plot

The parameter estimates were: $\alpha = -0.0092$, $\beta = 0.0802$ and $N_0 = 1.3081$.
The residual plot indicates a pattern to distribution of residuals.

Fig. 4.32. The fitted regression curve for treatment (VRBG-PETAH) and the corresponding residual plot

The parameter estimates were: $\alpha = 0.0686$, $\beta = -0.01754$ and $N_0 = 0.8369$.
The residual plot indicates a pattern to distribution of residuals.
indicates that the application of PA and PETA reduces the initial numbers of all populations of bacteria but affects the growth rate of the gram-negative bacteria more than the LAB.

The application of high pressure (PAH and PETAH) extended the lag phase of the total aerobic population in the time period of the study (Fig. 4.24). This indicates that the treatment has both an initial effect of reducing cell numbers but also a longer lasting effect of inducing sub-lethal injury. Recovery from this kind of injury is seen to take longer than from radiation damage. The same effect was seen in case of the LAB and gram-negative populations (Figs. 4.28 and 4.32). Treatment with irradiation also affected both the $N_0$ and $\beta_0$ values (Table 4.10). It is clear from the fitted curves (Fig. 4.23, 4.27 and 4.31) that the effect was primarily on the initial numbers and the slope of the log phase of growth, rather than the lag phase. Also the effect on final numbers was not as great as those achieved by high-pressure treatment.

The use of EDTA and Triton X-100 either increases the initial reduction in numbers in lactic-acid bacteria and/or effects a change in the growth rate of the population. In gram-negative bacteria the treatment extends the lag phase to a considerable extent. The effect of EDTA and Triton X-100 is most pronounced on the gram-negative cells. These chemicals therefore fulfilled the purpose for which they were included in the treatment i.e. extending the target range of the bacteriocin to include resistant cells, most of which were gram-negative. Also, when comparing treatments with respect to extension of shelf life, high-pressure treatment was most improved by the addition of EDTA and Triton X-100 (Fig. 4.32 and 4.24).

The studentized residual plots proved useful not only for the purpose of checking the fit of the model but also to indicate the nature of the deviation of the data from the fitted model. Most of the residual plots created indicated no distinct pattern to the distribution of the residuals. However regressions fitted to most of the data involving gram-negative populations showed a definite pattern to the deviations from the fitted model. For example, in Fig. 4.33, the curve fitted to treatment VRBG-PETAH shows just such a pattern: there is a greater positive and negative deviation from the fitted regression with time. This is to be expected in a system in which the constituent species of the test populations varies greatly from sample to sample. Though the various species may be susceptible to the treatment at the same level (thereby giving similar $N_0$ values), they grow at different rates thereby giving rise to the greater deviations from the fitted curve as growth proceeds with time. The lack of such a pattern in case of gram-positives may be due to the fact that they are highly susceptible to the treatments and that the surviving population consists of bacterial strains with similar growth rates and susceptibility.

**Effect of Treatments on Cooked Meat**

High-pressure treatment was not tested on cooked meat. Treatments incorporating irradiation had much lower numbers of total aerobic plate counts as well as lactic acid bacteria. Microbial numbers in control samples rose rapidly and steadily and reached spoilage levels (set at $10^6$ CFU/g) at 18 to 19 d. Bacterial counts of samples treated with PA also increased steadily after a lag of 9 d. Microbial populations of samples other than the control did not reach the set spoilage level. When treatments were compared for time taken to reach $10^2$ CFU/g, it was apparent that samples treated with PA reached that level about 8 days later than the controls.
Fig. 4.33. Effect of various treatments on total aerobic bacteria on cooked beef

Fig. 4.34. Effect of various treatments on lactic acid bacteria in cooked beef
Fig. 4.35. Effect of various treatments on the enterobacteriaeeae in cooked beef
Those treated with PETA reached that level almost 22 days later. Microbial numbers did not increase beyond $10^2$ CFU/g in the samples treated with PETA, PAI and PETAI. Hence, no significant differences between these treatments could be observed over the period of this study. Gram-negative populations in cooked meat were much lower than the total aerobic counts and lactic acid bacteria. This can be attributed to the fact that Gram-negative bacteria are much more heart-sensitive than Gram-positive bacteria. Figures 4.33, 4.34 and 4.35 illustrate the total aerobic, lactic acid spoilage and gram-negative bacterial populations as affected by the various treatments over time. Most of the microbial populations in the cooked meat study were at or below detection limits. Hence, a statistical analysis was not carried out on these data. However, the average plots show the same trends as the ones for raw meat.

**Conclusions**

The combination of bacteriocins with various chemical and physical anti-microbial treatments effected an extension in shelf life ranging from 5 d (in case of PA) to more than 15 d (in case of PETAH and PAH) in raw meat under conditions of temperature abuse. In cooked meat, treatment with bacteriocins and acid resulted in an extension of shelf life by a minimum of 5 d. The treatments PETAI and PAI did not reach levels of $10^2$ CFU/g after 28 days of storage.

Holzapfel *et al.* (1993) listed three major categories of drawbacks to the use of bacteriocins as food preservatives, adaptation (i.e., development of resistance in target organisms), metabolic activity (i.e., inactivation of starter cultures, deleterious effects on sensory qualities of food) and specific antibacterial factors (including binding and degradation by food components, presence of non-susceptible organisms). The above study has successfully addressed two of the three limitations with the experimental design. Combinations of two or more bacteriocins considerably reduce the frequency of formation of resistant mutants (Hanlin *et al.*, 1993). Also, studies indicate that the use of multiple ‘hurdles’ such as organic acid, heat treatment and salt along with bacteriocins tends to minimize the occurrence of resistant mutants (Martinis *et al.*, 1997). The production of large volumes of pediocin by the scale-up method described in Experiment 1 allows for the use of high levels of bacteriocin activity so as to successfully address the problems of protein binding. Also, heat treatment (cooking) can be used to denature bacteriocin-degrading proteases. Non-susceptible organisms can be targeted by agents/methods such as irradiation, high pressure and organic acids, or by bacteriocins themselves in combination with ion-chelators and detergents (Schved *et al.*, 1994a; Blackburn *et al.*, 1989). Lastly, the treatments applied involved the use of mostly food-grade chemicals (except Triton X-100). This greatly eases the development of formulations specific to individual types of food products that would have minimal negative effects on sensory qualities of the food.

Statistical analysis of data from these experiments indicated definite trends to the effect of various treatments on microbial sub-populations. There were differences in both initial reductions of numbers as well as inhibition of growth between treatments using irradiation and those using high pressure. The use of EDTA and Triton X-100 increased the initial reduction in bacterial numbers and also the lag phase of population growth.
especially in combination with high pressure. Statistical analysis also identified specific effects of treatment combinations such as the use of EDTA and Triton X-100 along with bacteriocins. The use of high-pressure treatment was most effective against gram-negative bacteria and tended to extend the lag phase of population growth, while the other treatments effected shelf-life extension by reduction of initial numbers and alteration of maximum growth rate. The fitting of non-linear regressions to the data enabled the identification of the specific growth curve parameters that were changed.

Considering the shelf-life extension reported in this study applies to samples stored under temperature abuse conditions, the shelf life at lower temperatures would be greater and exceed by far the objective of the study (15 and 20 days at 4-7°C for raw and cooked meat respectively). Also the experimental design and analysis of data report not only the end result in terms of shelf life but also the initial and storage effects of each treatment and the differences between treatments.
CHAPTER 5. GENERAL CONCLUSIONS

The aim of this research project was to develop a method to extend the shelf life of fresh and cooked meats by a minimum of 15 and 20 days respectively under conditions of refrigerated storage (i.e., 4-7°C). The use of bacteriocins was chosen as the first treatment to be studied. A scale-up method was developed for the production of large quantities of pediocin AcH. Studies indicated that inhibitory effect of this bacteriocin on the microflora of meat products was affected by several factors. The degradation of the protein by food enzymes, the occurrence of resistant microorganisms in meat and interactions with various food ingredients were identified as some of these factors. Organic acids, ion chelators, irradiation and high-pressure treatments were combined with bacteriocin in order to address these issues. The final step was the development of a combination of treatments that would provide optimum reduction in bacterial numbers and control of bacterial growth in the meat samples. The unique feature of this study was the extensive statistical analysis of the data in order to evaluate the results and also to identify mechanisms by which bacterial populations were affected by the treatments.

General Summary

The preliminary study involved testing a combination of crude pediocin AcH, acetic and lactic acids and immobilization in calcium alginate on the shelf life of raw beef at 4-7°C. The treatments were evaluated both with respect to initial reduction of bacterial numbers and the control of bacterial growth during storage. The data were analyzed by fitting linear regressions to growth curves of each treatment and using a t-test to test for significant differences in intercept and slope. Acetic acid was much more effective at reducing microbial numbers and delaying spoilage than lactic acid. Spoilage did not occur during the 5-week period of the study but data were insufficient to obtain an estimate of shelf life in case of these treatments. While pediocin alone did decrease the initial numbers of bacteria in the meat, it had little effect on controlling their growth over storage and so did not cause a shelf-life extension of more than 1.5 weeks. Also the combination of crude pediocin and acid was not significantly more effective than the use of that acid alone. Immobilization also did not extend the shelf life of the meat any more than the application of acetic acid alone.

In order to further address the limitations of bacteriocin application, further studies were conducted. As a first step, a simple method was developed to scale-up the production and partial purification of pediocin AcH to larger volumes (50 L of culture). This method used simple food grade materials and yielded approximately 50% recovery of bacteriocin activity. The semi-purified pediocin was then analyzed by SDS-PAGE to determine its purity and compared to other methods of purification, namely ethanol precipitation and ammonium sulfate precipitation. The semi-purified preparation contained far fewer impurities than the culture supernatant and samples prepared by other purification methods. The gels were also overlaid with a lawn of indicator cells to
confirm the activity of the bacteriocin band. A 2.6 Kda band of pediocin was identified by its clear inhibitory activity on the lawn of indicator cells.

A combination of pediocin and lactic acid was tested for combined activity against *Listeria monocytogenes* both in broth systems as well as in raw meat. The effect of salt concentration on this combined action of acid and bacteriocin in meat was then studied. Pediocin AcH was highly inhibitory to *Listeria monocytogenes* Scott A. Cells suspended in peptone water were much more susceptible to the bacteriocin than cells attached to meat. This anti-listerial activity of Pediocin was enhanced by the addition of lactic and acetic acids. However, increased salt concentrations adversely affected both the action of bacteriocin and the combined effect of pediocin and lactic acid. In the presence of 1.0 mM NaCl, more *Listeria* cells survived treatment with a combination of pediocin and acid than cells treated with pediocin alone. These results were validated by statistical analysis.

The next step was to characterize the microflora that survives treatment with bacteriocin. The MIDI-FAME system was used to identify the species of bacteria that survived bacteriocin treatment. The results indicated that high levels of bacteriocin (100,000 AU) eliminated most lactic acid bacteria, micrococcii and staphylococci in retail ground beef but had little effect on gram-negative spoilage bacteria such as *Pseudomonas* and *Acinetobacter*.

The loss of bacteriocin activity during storage was traced in raw and cooked meat. This loss of activity was measured at different storage temperatures. Bacteriocin activity was rapidly lost, presumably by degradation by proteolytic enzymes, in raw ground beef at around room temperature. The rate of activity loss was seen to be proportional to temperature, with activity persisting in frozen samples of the same ground beef for a period of up to 6 months. In cooked ground beef, where most of the enzymes have been denatured by heat treatment, the pediocin activity lasted for a longer period of time both under refrigeration and at 25°C. Here too an increase in storage temperature brought about an increase in rate of pediocin degradation. When samples were frozen for a period of time and then refrigerated to mimic conditions of abuse, the pediocin degraded more rapidly than in samples that were refrigerated throughout. The bacteriocin was seen to withstand heating at temperatures of 80°C. The protein also remained unaffected by e-beam irradiation at levels of 7.5 kGy and pressure of 10,000 psi. This indicated that these two treatments could be used in combination with pediocin AcH in a food preservation system.

Different combinations of ion-chelators (Maltol-80 and EDTA), Tween-80 and Triton X-100 were evaluated for their ability to enhance the anti-microbial activity of pediocin AcH against gram-negative pathogens, namely *Salmonella typhimurium* DT104 and *Escherichia coli* O157: H7. A combination of EDTA and Triton X-100 caused the maximum reduction of bacterial numbers and was chosen for use in the next experiment.

Finally, pediocin AcH and nisin used in combination with acetic acid, EDTA (an ion chelator), Triton X-100 (a detergent) and vacuum packaging, were much more effective against Gram-negative bacteria than when used with acetic acid alone. Treatment of beef cubes with the above-mentioned combination of
antimicrobials and high pressure was by far the most effective at prolonging shelf life of the meat. Combining these chemical and biological agents with e-beam irradiation was also effective at inhibiting spoilage microflora, but not as much as high-pressure treatment. The use of pediocin, nisin and irradiation on cooked meat was highly successful in controlling microbial growth for up to 27 days even when stored at conditions of temperature abuse.

The statistical analysis of data generated by the various experiments was useful in identifying the specific nature of changes in bacterial populations. Irradiation treatment mainly reduced the initial numbers of total microflora as well as the rate of growth while high hydrostatic pressure treatment primarily increased the lag phase of the population growth, and also reduced initial numbers of gram-negative bacteria. The use of EDTA and Triton X-100 extended the target spectrum of pediocin AcH especially when used in combination with high pressure, thereby rendering it more effective against gram-negative species. The combination of pediocin, nisin, EDTA, Triton X-100 and high pressure was effective in delaying spoilage of raw beef for a minimum of 20 days of storage under modified atmospheres at abuse temperatures of 15°C. In case of product cooked for 3 min at 80°C, a combination of pediocin, nisin and irradiation was effective in delaying spoilage of raw beef for a minimum of 28 days of storage under modified atmospheres at abuse temperatures of 15°C. Microflora in both the raw and cooked meat mentioned above did not reach spoilage levels during the period of the study (20 and 27 days respectively).

**General Discussion**

This study was aimed at delaying the spoilage of meat products. The use of new technologies such as irradiation and high pressure is very effective in controlling the growth of microflora, but are limited by various considerations such as effects of sensory qualities of meat, equipment design, etc. Bacteriocins were chosen as the primary agent of preservation because they have little effect on the sensory attributes of food. Use of bacteriocins in a commercial scale is limited by the cost of production of the protein. This issue was addressed developing a scale-up method for the production of large volumes of semi-purified bacteriocin using food grade chemicals and processes. Other treatments were included in order to address the drawbacks to bacteriocin application and further extend the shelf life of meat.

This study has shown that bacteriocins can indeed be effective antimicrobial agents but only under very specific circumstances. Though their proteinaceous nature and small size are desirable traits, they render the bacteriocins susceptible to proteolytic enzymes in the meat. And while pediocin AcH and nisin are very effective against several Gram-positive organisms including *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus sterothermophilus*, *Bacillus coagulans*, *Lactococcus* spp. and *Lactobacillus* spp., several species of spoilage organisms such as *Pseudomonas* and *Acinetobacter* spp. are resistant. These bacteriocins therefore have to be used in combination with other treatments. The combined action of ion chelators (to disrupt the LPS layer of
resistant Gram-negative cells), detergents (to stabilize the bacteriocin in solution) and high pressure (to cause injury to resistant cells) offers an effective new method of preservation of meat. When considering the use of bacteriocins in meat products, the salt, fat and protein content of the product must be taken into consideration as well as its pH and the temperature of processing and storage. Knowing the duration of time for which bacteriocins are likely to survive in a product would prove valuable in determining preventive action should any temperature abuse of the product occur.

The treatment combination developed in this study successfully extended the shelf life of raw meat by a minimum of 20 days under temperature abuse conditions. In case of cooked meat, some of the treatments kept the levels of microflora to below 1 log unit per gram over a period of 27 days under temperature abuse conditions. This by far exceeds the objective of the experiment.

A unique feature of this study has been the extensive use of statistics to analyze the data on population growth dynamics. There are no reports in the literature of studies that have used a similar approach. The dynamics of bacterial sub-populations was seen with the use of statistical analysis. Gompertz equation was used to fit a non-linear regression model to the bacterial growth pattern of each treatment. The fitted curves revealed differences in the effect of various treatments on the growth of bacterial populations of the samples. Such analyses could be used in the predictive modeling of mixed microbial cultures in various foods under certain environmental conditions (e.g., temperature abuse). They could also be used to study specific effects of anti-bacterial agents and food preservatives on bacterial populations. Statistical analysis can also be used to identify the nature of interactions between various agents.

With increasing demand for 'all natural' products and the introduction of high pressure processing into the US food industry, developing treatment combinations similar to those described in this dissertation would be easy. The successful use of Nisin as a preservative in several types of food is an indication of the potential of bacteriocins as food preservation agents. Research is being conducted on the identification, commercial scale production, purification and application of several bacteriocins. Bacteriocins may also find application in other areas, such as development of probiotics, environmental sanitation and medicine.

**Recommendations for Future Research**

1. **Food safety application**: The use of bacteriocins against spoilage and pathogenic bacteria in various meat products warrants investigation. Of particular importance is the problem of post processing contamination of cooked, ready-to-eat products such as frankfurters and hot-dogs. Studies should be conducted on the inhibition of organisms such as *L. monocytogenes* and other pathogens by bacteriocins that have been included in various meat products before or after thermal processing.

2. **Food safety application**: The use of bacteriocins in combination with high pressure on foods which are adversely affected by heat treatment such as juices, guacamole, egg products, dairy products, etc warrants
investigation. Preliminary studies indicate that bacteriocins can be used to target spores in these products that may be resistant to high pressure processing.

3. **Environmental microbiology**: Environmental isolates of bacteria should be screened for of broad-spectrum bacteriocins effective against a different range of microorganisms. A broad-spectrum gram-negative bacteriocin could find extensive use in both food and non-food systems.

4. **Protein engineering**: In order to better understand the action of broad-spectrum bacteriocins, the specific molecules involved in the adsorption and insertion of the protein onto the cell surface (both specific and non-specific receptors) have also to be characterized. The structure of these molecules may provide clues as to what domains are involved in specific and non-specific binding of bacteriocins. Such information may help in the engineering of other bacteriocins with broader inhibitory spectra.

5. **Industrial fermentation**: Though nisin is used extensively in the food industry, it is still an expensive protein to produce in pure form. The development and optimization of large-scale fermentations to produce bacteriocins, as well as downstream processes for their purification are essential for the establishment of bacteriocins as food preservatives. This includes researching alternative and cost effective substrates, optimizing bio-reactor design, and improving efficiency of purification and recovery.

6. **Genetic engineering**: The construction of strains of starter culture bacteria that produce multiple bacteriocins is being explored for use in the dairy and meat industry. Strains that produce both Gram-positive and Gram-negative bacteriocins would have a much broader spectrum of action against a variety of spoilage and pathogenic bacteria.

7. **Molecular biology and biochemistry**: The mechanism of resistance to specific bacteriocins must be elucidated to better develop physical and chemical treatments to be used in combination with these proteins. For example, if resistant cells lack the appropriate receptor for the bacteriocin, another method (such as high pressure) can be used to deliver the bacteriocin to its target site. The bacteriocin itself could be hybridized with one that does bind to the cell surface.
REFERENCES


Dickson, J. S., 1990. Surface moisture and osmotic stress as factors that affect the sanitizing of beef tissue surfaces. J. Food Prot. 53: 674.


Hoover, D. G. 1996. A combination of bacteriocins, high pressure and heat was used to achieve significant reductions in the numbers of pathogens such as S. aureus, L. monocytogenes, S. typhimurium, and E. coli O157:H7.


Houtsma, P. C., J. C. de Wit, and F. M. Rombouts. 1996. Minimum inhibitory concentration (MIC) of sodium lactate and sodium chloride for spoilage organisms and pathogens at different pH values and temperatures. J. Food Prot. 59: 1300-1304.


Ockerman, H. W., R. J. Borin, V. R. Cahill, N. A. Parrett, and H. D. Hoffman. 1974. Use of acetic and lactic acid to control the quantity of microorganisms on lamb carcasses. J. Milk Food Technol. 37:203-204.


ACKNOWLEDGEMENTS

There are many people who have contributed to making my years at Iowa State happy and fulfilling. First and foremost, I thank my advisor, Dr. James Dickson, for his guidance, encouragement and for being a role model in more than just academics.

I also would like to thank the members of my program of study committee, Dr. Alicia Carriquiry, Dr. Alan Dispirito, Dr. Bonita Glatz, Dr. Dennis Olson, and Dr. Joseph Sebranek, for their advice, feedback and help. I am indebted to Dr. Mike Johnson, Dr. Rama Nannapaneni and Marlene Janes of the Food Safety lab at the University of Arkansas–Fayetteville, for taking the time and effort to help me through some of the aspects of my project.

I am also grateful to Mike Holtzbauer, James O’Brien, John Strohl, Benjamin Voss and Dr. Larry Halverson and his laboratory staff for helping with various parts of my project.

I would also like to thank Steve Neibuhr and Robert Hubert for being friends, teachers, troubleshooters and so much more. I am also grateful to my friend and mentor, Dr. Rodrigo Tarté, for his guidance in the early stages of my research. My special thanks to all my office-mates and colleagues, especially Shannon Green and Chih-Ming Chen, for their advice and support in research, Chi-Ching Foong for her assistance and guidance in the writing of this dissertation, and Sandip Sinharay for his invaluable assistance with statistical analysis. To Ann Hetland, Simi Venkatagiri and Val Evans in the Office of the Department of Microbiology, I give my heartfelt thanks for keeping my academic life organized, and providing tremendous support. I also thank my friends Dr. Surya Mallapragada and Rohini Ramaswamy for their unwavering support and assistance.

Most importantly, I thank my parents and my grandmother, for believing in me, never expecting anything less of me, and most of all, for teaching by example.